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THE CHEMISTRY AND UTILIZATION OF CYCLOPENTADIENE

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Cyclopentadiene is a low-boiling cyclic diolefin which has been found among the products resulting from the pyrolysis of organic compounds. Commercially important quantities can be recovered from the distillates produced in carbonization of coal, particularly the forerunnings from coke-oven light oil. The chemistry of cyclopentadiene is reviewed with particular reference to its industrial application. Not only is this substance of interest for the production of resins, but it can also be the starting point for the synthesis of a large number of other organic products, some of which should find use in a variety of fields. On standing, cyclopentadiene spontaneously polymerizes to dicyclopentadiene, a compound solid at ordinary temperatures. Because the latter is readily reconverted to the monomer, it forms a more convenient form in which to handle cyclopentadiene. The properties of dicyclopentadiene are likewise reviewed.

I. INTRODUCTION

Dienes are vitally important to our national life at present, because they can be utilized to produce synthetic products which may replace natural rubber. This family of compounds is consequently receiving much attention in order to find members which will improve the properties of the new rubbers and will be useful in the synthesis of resins. A very interesting diene is cyclopentadiene, which not only possesses the reactive, conjugated, double-bond structure, but also contains an active methylene group. Already from cyclopentadiene a large number of polymers and derivatives have been prepared, some of which are proving of commercial value in the field of synthetic resins. A few of the more significant reactions will be emphasized at this point.

Cyclopentadiene can be polymerized in the presence of catalysts to produce rubber-like compounds. The reaction is analogous to the production of synthetic rubbers from other conjugated dienes. The present sources of cyclopentadiene are not sufficiently productive at this time to furnish any appreciable volume of synthetic rubbers for use in tires. Cyclopentadiene, however, may find application in the rubber program as a copolymer which will modify the properties of other rubbers.

By the heat polymerization of cyclopentadiene there are produced structures which are characterized by a chain of hexagonal carbon rings, each containing a bridging methylene group and terminated at one end by a pentagonal ring. Dicyclopentadiene, which forms spontaneously in cyclopentadiene at ordinary temperatures, is the simplest member of the series. Its structure is similar to that of indene except for the methylene bridge and two extra hydrogens. The

¹ Multiple Industrial Fellowship sustained by the Carnegie-Illinois Steel Corporation, Pittsburgh, Pennsylvania.

highest polymers are waxy solids, the application of which—to replace natural waxes, for instance—has not as yet been explored.

The preparation of a very large number of derivatives by the addition of cyclopentadiene and an olefin by the Diels–Alder reaction is possible. This synthesis enables the preparation of such important compounds as terpene, camphor, and partially hydrogenated benzene derivatives. Thus, norcamphor has been produced from cyclopentadiene and acrolein. The compounds formed by the heat polymerization of cyclopentadiene are also examples of Diels–Alder reaction products. Another is the addition of maleic anhydride to cyclopentadiene. The product, *cis*-3,6-endomethylene- Δ^4 -tetrahydrophthalic anhydride, is similar in structure to phthalic anhydride, except for the addition of a methylene bridge and two extra hydrogens. It can be used to produce alkyd resins. High-boiling esters of the acid have been recommended as plasticizers. The complex quinones produced by the addition of cyclopentadiene to quinones may prove of interest in the dye industry.

By the condensation of cyclopentadiene with aldehydes and ketones highly colored fulvenes are produced. These may find application in pharmaceuticals. Barbiturates prepared from cyclopentadiene and malonates already have important pharmaceutical applications. Derivatives, such as alcohols, amines, and halogenated compounds, can be readily synthesized from cyclopentadiene.

These reactions render cyclopentadiene of especial interest as a raw material in the resin, pharmaceutical, and synthetic organic chemical fields. The surface has scarcely been scratched; further study of the diene and of its derivatives will doubtless extend the number and the importance of the industrial applications. Supplies of the compound can be made available as the needs arise.

II. SOURCE AND PRODUCTION

A. PYROLYSIS OF HYDROCARBONS

Cyclopentadiene is one of the many compounds formed during the pyrolysis of hydrocarbons at high temperatures, and both it and its dimers have been separated from the volatile products of such reactions. In the carbonization of coal, the tar, the light oil, and the coke-oven gas have yielded these two compounds. Kraemer and Spilker (140) in 1896 first reported the presence of cyclopentadiene in coal-tar fractions.

1. Benzol forerunnings

The presence of cyclopentadiene in the forerunnings fraction produced during the distillation of coke-oven light oil has been frequently reported (50, 56, 107, 108, 143, 188, 230, 239). A forerunnings fraction completely resinified by sulfuric acid was found by Kruber (142) to consist mostly of cyclopentadiene; the residue appeared to be dicyclopentadiene. In some crude light oils Gillies (93) found as much as 1.2 per cent of cyclopentadiene. At present benzol forerunnings is probably the largest potential source of cyclopentadiene which

is receiving attention. From the experience of the authors it is estimated that the concentrations in light oil will be between 0.5 and 1 per cent by volume, equivalent to between 0.1 and 0.2 pound of cyclopentadiene per ton of coal carbonized. Although such a yield appears small, the potential volume available from the coal coked annually in the United States would represent many thousand gallons of cyclopentadiene; its recovery, particularly in the larger plants, would frequently prove practical.

Because cyclopentadiene spontaneously polymerizes at ordinary temperatures to dicyclopentadiene, which boils at approximately 170°C., the latter will be present in the higher-boiling fractions of the light oil, particularly after standing, or in coal-tar fractions. No steps appear to have been taken as yet towards the recovery of dicyclopentadiene from these higher-boiling fractions, but Carmody and coworkers (49, 50, 51, 52) have produced resins by polymerizing the dicyclopentadiene with other compounds present in the fractions. The solvent-naphtha fraction of the light oil thus represents another potential source of cyclopentadiene, provided recovery methods can be developed.

2. Coke-oven gas

Ross and Race (188) found cyclopentadiene in both coal gas and water gas. They separated cyclopentadiene derivatives from the oils produced by brominating the gases, and identified cyclopentadiene in the forerunnings from the anthracene oil which had been used to wash naphthalene from the gas. In France the Bethune Company recovered 35-45 pounds of cyclopentadiene per 1000 tons of coal by liquefaction and fractional distillation of coke-oven gas, according to Horelois (111). Fractional distillation of the gas does not appear to be a very efficient method for separating such a small fraction, but as yet no better method has been disclosed.

3. Carburetted water gas

When petroleum oil is cracked to carburetted water gas, cyclopentadiene is found among the products and may be recovered from the tar, drip oil, or condensates produced by refrigeration of the gas, according to Ward (230). A fraction which he distilled from water-gas condensates contained 18 per cent of cyclopentadiene, as well as isoprene, piperylene, pentenes, and other hydrocarbons. Such condensates can apparently become another useful source of cyclopentadiene.

4. Hydrocarbon cracking

Cyclopentadiene has been found in many products obtained by the pyrolysis of natural gas, petroleum, and its fractions. It was first isolated in 1891 by Étard and Lambert (81) from oil gas. Cyclopentadiene was identified in the products from the cracking of Persian natural gas by Birch and Hague (39). Volzhinskii and Shegeglova (228) reported that 30-35 per cent of the 28-50°C. fraction which they obtained in the vacuum cracking of kerosene consisted of

dienes, and that the greater part was cyclopentadiene. Small amounts of cyclopentadiene were produced during the cracking of a Pennsylvania gas oil at 950°C. and 175 mm. pressure, according to Tropsch and coworkers (224). Potolovskii and Vimberg (177) found that a low-boiling fraction obtained during the pyrolysis of a Russian gas oil contained 5.5–6.0 per cent cyclopentadiene, as well as some isoprene. The fraction boiling between 27° and 50°C. produced by the pyrolysis of another gas oil was reported by Dedusenko (60) to contain 3.5–3.7 per cent of cyclopentadiene. In the fraction boiling at 30–45°C. obtained from a gasoline produced by vapor-phase cracking at 570°C., Moor and Katzman (157) found 6–8 per cent of cyclopentadiene.

When Frey and Hepp (90) cracked the lower paraffins, other than methane, at 850°C., cyclopentadiene was one of the principal products. Cyclopentadiene was present in the oil fraction boiling below 200°C. which Cambron and Bayley (47) produced by cracking propane at 800–810°C. in alloy-steel tubes under conditions of turbulent flow. Kazanskiĭ and Plate (121) separated a little cyclopentadiene from the products obtained by pyrolyzing cyclopentane at 650°C. In the treatment of ethylene hydrocarbons at elevated pressures and temperatures Dunstan and coworkers (73) produced some cyclopentadiene.

5. *Pyrolysis of phenol*

When Ruhemann (189) heated phenols at 750°C. in contact with quartz, glass, or pumice, carbon monoxide split off and the nucleus rearranged to form cyclopentadiene. In 1886 Roscoe (187) first found dicyclopentadiene in the products obtained by the pyrolysis of phenol.

B. RECOVERY FROM OILS

The dimerization of cyclopentadiene was utilized by Ward (230) for its recovery from oil fractions containing other polymerizable dienes. By heating the mixture to a temperature in the neighborhood at 100°C., under a pressure sufficient to maintain the larger part of the reaction mixture in the liquid phase, dicyclopentadiene was produced. Dimerization takes an appreciable time, and in one example Ward heated the oil at 92°C. for 24 hr. The dicyclopentadiene was separated from the product by fractional distillation under vacuum, using a pressure of 25 mm. of mercury absolute. A product containing 95–97 per cent of dicyclopentadiene was obtained.

In the Annual Report of the Mellon Institute for 1941–42 (238) there is mentioned a process, developed by the authors, for the recovery of cyclopentadiene from benzol forerunnings, which also depends on the formation and recovery of dicyclopentadiene.

Dicyclopentadiene is readily reconverted to the monomer by distilling the former under atmospheric pressure. By maintaining the top temperature of the fractionating column of their still at 40–41°C., Kistiakowsky and coworkers (131) obtained a distillate of cyclopentadiene alone. As dicyclopentadiene boils at approximately 170°C., it was refluxed to the still kettle. Dicyclopentadiene

thus represents a convenient form in which to handle and store cyclopentadiene. Ward (230) recommended that it be stored in an atmosphere free from oxygen, such as nitrogen, to avoid the formation of peroxides. A number of investigators have used the depolymerization of dicyclopentadiene to cyclopentadiene as a means for obtaining a high-grade material for use in experimental work (131, 159).

The Diels-Alder reaction of cyclopentadiene with maleic anhydride has been utilized for separating cyclopentadiene from coke-oven light oil. The crystalline *cis*-3,6-endomethylene- Δ^4 -tetrahydrophthalic anhydride was separated from the unreacted oil, according to the patent of the I. G. Farbenindustrie (114). Morrell and coworkers (156) used the reaction for removing cyclopentadiene from gasoline.

Tyutyunnikov (226) used maleic anhydride, benzoquinone, α -naphthaquinone, or a similar substance for separating the cyclopentadiene from light-oil fractions. Because indene, coumarone, and related substances react with the same reagents, the cyclopentadiene adduct was recovered with them as a high-melting resin. Potolovskii and Vimberg (177) also employed benzoquinone for removing cyclopentadiene from a light-oil fraction. Cyclopentadiene cannot be recovered directly from the maleic anhydride adduct, but it may prove practicable to decompose the quinone derivatives with recovery of the reagents.

C. SYNTHESIS OF CYCLOPENTADIENE

Cyclopentadiene has been synthesized by the dehydrogenation of cyclopentane, according to Morrell (155). A 9 per cent yield was obtained by Grosse and coworkers (98, 99) by passing cyclopentane, at 500–600°C. and 0.25–1 atmosphere pressure, over dehydrogenating catalysts such as Al_2O_3 impregnated with oxides of chromium, molybdenum, and vanadium. The time of contact was 0.5–6 sec. Frey (89) found 2.7 per cent of cyclopentadiene in the products of the dehydrogenation of cyclopentane at 575°C. in a silica tube.

Cyclopentadiene has been prepared by boiling 1,2-dibromocyclopentane at 110°C. for several days with ethylene glycol and potassium (159). The same investigators, Mousseron and Granger (160), also obtained it by heating either 2-methoxy- or 2-ethoxy-cyclopentanol with sulfuric acid.

Cyclopentadiene was synthesized by Zelinskiĭ and Levina (245) from cyclopentanone. The latter was reduced, using hydrogen and an osmium catalyst, to cyclopentanol, which in turn was dehydrated to cyclopentene by heating with anhydrous oxalic acid. The dibromide produced from the cyclopentene in a chloroform solution was finally converted to cyclopentadiene by heating at 180°C. with an acetic acid-sodium acetate mixture.

III. PHYSICAL PROPERTIES

A. PHYSICAL CONSTANTS

Values for the melting and boiling points, the density, and the refractive index of cyclopentadiene are collected in table 1.

The following densities for cyclopentadiene at different temperatures are given by Raistrick and coworkers (178):

TEMPERATURE	d^4
°C.	
0	0.8235
10	0.8131
20	0.8021
25	0.7966
30	0.7914

Barrett and Burrage (32) have determined the vapor pressures of cyclopentadiene at different temperatures. When the reciprocal of the absolute temperature was plotted against $\log_{10} P$, a straight line was obtained in the range

TABLE 1
Physical constants of cyclopentadiene

MELTING POINT °C.	BOILING POINT		DENSITY		INDEX OF REFRACTION
	°C.	At mm. of mercury absolute			
-85 (202a)	40	760 (202a)	0.8026	d_4^{20} (231)	1.4429 n_D^{20} (231)
	41	760 (140)	0.803	d_4^{20} (81)	1.4398 $n_D^{19.5}$ (245)
	41.5-42	760 (209)	0.7983	$d_4^{19.5}$ (245)	1.4446 $n_D^{18.6}$ (140)
	41.5	760 (77)	0.80475	$d_4^{18.6}$ (140)	1.44627 $n_D^{16.1}$ (31)
	42.5	760 (81)	0.8070	$d_4^{18.1}$ (31)	1.44632 $n_D^{16.1}$ (31)
	41	757 (245)	0.8085	$d_4^{16.1}$ (31)	
	40.2-40.8	757 (31)	0.8071	$d_4^{15.7}$ (77)	
	40-41	755 (103)	0.81500	d_{15}^{15} (140)	
	40	715 (220)	0.8083	$d_4^{14.1}$ (77)	
	40.83	772 (131)	0.8228	d_4^0 (30)	

0-15°C. From this curve, the following two values have been read: a pressure of 250 mm. of mercury at 12°C., and 44.7 mm. at -22.9°C.

Additional physical constants are as follows:

Dispersion $\times 10^4$ ($n_F - n_C$).....	132 (231)
Specific dispersion $\left(\frac{n_F - n_C}{d}\right)$	164 (100, 231)
Heat of vaporization per gram mole.....	7 kg.-cal. (234)
Heat of combustion per gram mole of cyclopentadiene.....	847.8 kg.-cal. (127)
of gaseous cyclopentadiene.....	707 $\pm$ 7 kg.-cal. (234)
Heat of formation from carbon and hydrogen per gram mole:	
Calculated from heat of combustion.....	1153 $\pm$ 7 kg.-cal. (234)
Calculated from bond energies.....	1152 $\pm$ 7 kg.-cal. (234)

Cyclopentadiene in benzene gives an abnormal depression of the freezing point, according to Garelli (91), probably owing to the formation of a tightly bound solution, but dicyclopentadiene, its dimer, affords normal values.

The spontaneous ignition point of cyclopentadiene in oxygen was found to be $510^{\circ}\text{C}.$, and in air, $640^{\circ}\text{C}.$ by Zerbe and Eckert (246), using the drop method. Although in agreement on the value in air, Tausz and Schulte (211) obtained $414^{\circ}\text{C}.$ in oxygen.

The octane number, as reported by Lovell and coworkers (148), was greater than 100; the aniline equivalent, 34; and the critical compression ratio, 10.9. Addition of tetraethyllead to cyclopentadiene was shown by Campbell and coworkers (48) to have a negative effect on the critical compression ratio.

B. SPECTRA

1. *Infrared*

The absorption spectra of fresh and polymerized cyclopentadiene have been studied in the infrared range by Lambert and Lecompte (144). In particular in the region of 700 cm.^{-1} the polymer shows a plurality of strong absorption bands. The strong band at 961 cm.^{-1} in the fresh material is displaced to 930 in the polymerized cyclopentadiene, and strong bands at 1237 and 1310 cm.^{-1} in the former have disappeared in the dimer.

2. *Ultraviolet absorption*

Pickett and coworkers (172) have found that in the ultraviolet range between $32,000\text{ cm.}^{-1}$ and $66,000\text{ cm.}^{-1}$ the vapor shows an intense absorption band with a maximum at $43,000\text{ cm.}^{-1}$, on which are superimposed narrow diffuse bands with maxima at $38,800\text{ cm.}^{-1}$, $39,650\text{ cm.}^{-1}$, $40,400\text{ cm.}^{-1}$, and $41,170\text{ cm.}^{-1}$, and a group of sharply defined bands beginning at $40,000\text{ cm.}^{-1}$. In hexane solution, the cyclopentadiene absorption maximum has approximately the same intensity as for the vapor but is shifted to $41,800\text{ cm.}^{-1}$ and has but two narrow strong bands. The presence of such an intense absorption band with characteristic, superimposed, narrow bands makes it possible to identify cyclopentadiene in minute traces and to estimate its concentration in mixtures of other hydrocarbons.

In the Schumann region there is a prominent group of narrow sharp bands between $50,350\text{ cm.}^{-1}$ and $54,000\text{ cm.}^{-1}$. Pickett and coworkers reported twenty-six bands in this region. Nine of the stronger bands have been recorded by Scheibe and Grieneisen (193). The band maxima agree within experimental error.

The measured frequencies of Price and Walsh (177a) are less by about 50 cm.^{-1} than those of Pickett. Two main frequency differences are involved,—one about 1450 cm.^{-1} and the other about 480 cm.^{-1} . Price and Walsh ascribed the first to a valence vibration in the $\text{C}=\text{C}$ bonds and the second to a twisting of the methylene group.

3. Raman spectra

Cyclopentadiene was among the five-membered rings the Raman spectra of which were studied by Reitz (184) by means of models. Calculated frequencies were compared with the experimental ones and with the frequency changes as a function of symmetry properties. He found no evidence of an abnormal behavior of the C=C bond. A number of lines, most of which were depolarized, have been recorded by Reitz (183, 185). The results are in modest agreement with those of Truchet and Chapron (225), Lambert and Lecompte (144), and Kohlrausch and Seka (135, 136). The particular feature in connection with cyclopentadiene is the shift $\Delta\nu$ 1500 attributable to the ethylenic linkage. While this large decrease is not analogous to that observed with the allenes, it is much lower than that seen in other unsaturated cyclic hydrocarbons (106).

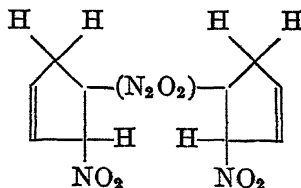
The molecular spectrum of cyclopentadiene has been studied by Mulliken (161, 162, 163), and an electron-diffraction investigation has been made by Schomaker and Pauling (194).

IV. CHEMICAL PROPERTIES

A. REACTIONS WITH MINERAL ACIDS AND ALKALIS

Cyclopentadiene reacts explosively with charring with concentrated sulfuric (140, 195, 237) and fuming nitric acids (140). Dilute acids resinify it, and soluble alkalis have a similar effect (140, 237). Potassium hydroxide in alcohol resinifies cyclopentadiene with vigorous evolution of heat (140). Kraemer and Spilker (140) isolated a compound, $C_{10}H_{12}(SO_3H)_2$, which was produced by the action of sulfuric acid on cyclopentadiene. Deep blue, high-molecular-weight products were prepared by treating cyclopentadiene with concentrated sulfuric acid at $-80^\circ C.$, according to Staudinger and Bruson (204). By addition of nitric acid to cyclopentadiene in chloroform Weger (237) obtained a nitrosate.

When Wieland and Stenzl (242) passed a current of nitric oxide gases through cyclopentadiene dissolved in ethyl ether, finely crystalline, pale yellow flocks were formed. They changed into a sticky brown mass, without loss in weight, when separated from the solution and allowed to stand for about three-quarters of an hour. It was concluded that the final product was cyclopentadiene pseudonitrosite:



B. HALOGEN DERIVATIVES

1. Addition of halogens

Halogens and halogen acids add readily at the unsaturated carbon linkages of the cyclopentadiene molecule. By such additions a series of halogenated

derivatives have been prepared which range, in the case of chlorine, from monochlorocyclopentene to tetrachlorocyclopentane. Addition of a halogen takes place in two steps: first, the union of one molecule to each molecule of cyclopentadiene, to form $C_5H_6X_2$; then addition of the second halogen molecule. The reactions, which are vigorous, should be performed at a low temperature and in an inert solvent (140). According to Grosse and Wacker (100), the theoretical bromine number of cyclopentadiene is 485.

Thiele (220) added bromine slowly to cyclopentadiene dissolved in chloroform to produce *trans*-1,4-dibromocyclopentene-2. Farmer and Scott (85) prepared both the *cis*- and the *trans*-isomers by brominating at -15°C . in *n*-hexane solution. In one experiment the yield of the solid *trans*-isomer amounted to 40 per cent, but it could be varied by changing the proportion of hexane. The colorless liquid left after removal of the crystals contained 1,2-dibromocyclopentene-3 and a small amount of *cis*-1,4-dibromocyclopentene-2. By treatment with potassium permanganate, the bromopentenes were oxidized to the corresponding dibromoglycols, which in turn were oxidized with dilute chromic acid to dibromoglutaric acid.

Rapid addition of the bromine favored formation of *cis*-1,4-dibromocyclopentene-2, according to Mayes (151), who brominated cyclopentadiene at -25°C . in chloroform. At the same time a considerable quantity of tetrabromocyclopentane was formed. Solid *trans*-1,4-dibromocyclopentene-2 was precipitated slowly from the liquid *cis*-isomers on standing, but on slow distillation the crystals reverted to the *cis*-form. The corresponding cyclopentadienediols were formed by heating the dibromo compounds with potassium acetate and acetic acid to produce the diacetates, and saponifying the latter with alcoholic hydroxides.

Schultze (195) observed that the difficulties he encountered in brominating both double bonds of cyclopentadiene were due to a rapid autooxidation prior to the reaction with bromine. Minute traces of the oxidation product had an enormous influence, and he concluded that bromination was a chain reaction. He described procedures for the quantitative bromination of the two double bonds in both cyclopentadiene and dicyclopentadiene either by titrating *in vacuo* or by introducing the cyclopentadiene into an excess of free bromine.

On addition of bromine in acetic acid to cyclopentadiene Staudinger and Brunson (204) obtained a deep blue, insoluble compound corresponding to the formula $C_{10}H_{11}CH_2COBr$; when heated it decomposed without melting with evolution of hydrogen bromide. Pauly and coworkers (169) reported that cyclopentadiene would slowly add close to two atoms of iodine from a tenth-normal iodine solution.

2. Addition of hydrogen halides

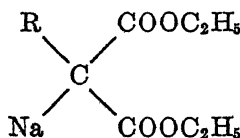
Dry hydrogen chloride, passed into a solution of cyclopentadiene in an inert solvent, such as toluene and chloroform, at low temperatures (-15°C . to -40°C .), added with the production of cyclopentenyl chloride (95, 140, 170). This compound decomposed on standing, with resinification and evolution of

hydrogen chloride. It should be stored in the dark at low temperatures or, better, used at once (95, 140, 165, 170).

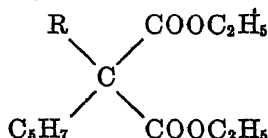
The halogenated derivatives of cyclopentadiene are interesting starting points for the synthesis of a large number of organic derivatives, including amines, alcohols, and thiocyanates. The preparation and application of these derivatives have so far received only limited attention. Ammonium hydroxide reacted with cyclopentenyl chloride to produce cyclopentenylamine and small amounts of an alcohol, and all primary and secondary bases react in a similar manner (140); e.g., by the addition of aniline at a temperature below 10°C., Noeldechen (165) obtained anilidocyclopentene. By the addition of chlorine to cyclopentenyl chloride at low temperatures Kraemer and Spilker (140) produced trichlorocyclopentane, which in the cold was resistant to concentrated sulfuric or nitric acid. Bases reacted with it slowly, while chlorine or hydrogen chloride split off.

3. Preparation of barbiturates

An application for the cyclopentenyl halides, which is suggestive to the pharmaceutical industry, is the preparation of barbiturates,—compounds with important sedative, analgesic, and hypnotic properties. Some of the barbiturates have also been used in the preparation of perfumes. The cyclopentenyl chloride is combined with the sodium compound of a malonic ester,



where R can be either hydrogen, an aryl group, or an aliphatic group, to produce the corresponding cyclopentenylmalonic ester:



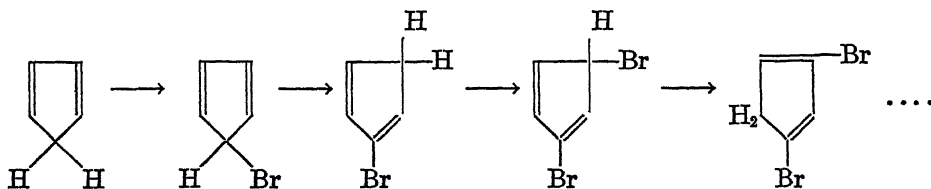
The preparation and properties of a number of barbiturates from these derivatives have been described by Chaux (54, 55) and by Horelois (111).

Noller and Adams (166) reacted a malonic ester and sodium alcoholate at 45–50°C., and then slowly added the cyclopentenyl chloride to the product at about 5°C. The cyclopentenylmalonic ester was saponified to form the acid and then decomposed by heating to produce the Δ^2 -cyclopentenylacetic acid, a homolog of chaulmoogric acid which is of interest in the treatment of leprosy, according to Perkins and Cruz (170). Amides and ureides, as well as esters of cyclopentenylmalonic acid, have been prepared by the same reaction (181).

4. Action of hypohalites

The active hydrogen of cyclopentadiene has been replaced by treating the compound with an aqueous alkaline solution of a hypohalite. Strauss and co-

workers (210) observed that the replacement progressed until all six hydrogens were eliminated. When a solution of cyclopentadiene in petroleum ether was shaken with hypobromite, crystals of hexabromocyclopentadiene began to separate in 10 min. The yield exceeded 50 per cent. The course of the reaction was represented as follows:



By treatment with hypochlorous acid, the cyclopentadiene in a light-oil fraction produced in the cracking of a gas oil was separated as the chlorohydrin. Dedusenko (60) then distilled the aqueous solution of the chlorohydrins with potassium hydroxide and from the alkaline residue separated *cis*-cyclopentadiol by extraction with ether.

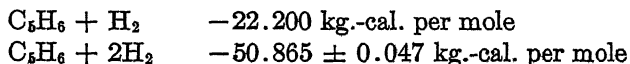
C. REDUCTION

Cyclopentadiene reduces an ammoniacal silver solution with production of a silver mirror (188). Low yields of cyclopentene or cyclopentane were obtained by the action of zinc and hydrochloric acid on cyclopentadiene in alcoholic solution (140).

Because dilute acids and alkalis resinify cyclopentadiene, Weger (237) has recommended its reduction by the use of finely divided nickel, and with this catalyst Eijkman (78) has obtained cyclopentane. Its solvent properties have attracted some industrial attention. Kazanskiĭ and Glushnev (120) have produced cyclopentene by reducing cyclopentadiene with calcium ammonia, $\text{Ca}(\text{NH}_3)_6$. By passing the diene with hydrogen over platinum-charcoal at 160°C ., Zelinskiĭ and Levina (245) have prepared cyclopentane and tricyclopentadecane.

Foresti (88) determined an activity curve for the rates of hydrogenation of cyclopentadiene in the presence of platinum as a function of the apparent pH of the medium in which the reaction was carried out. The form of the curve was typical of that for monoolefins. It showed a slight rise with increase in the apparent pH in acid and a sharp drop in alkaline media.

The following heats of hydrogenation for cyclopentadiene at 82°C . were reported by Kistiakowsky and coworkers (131):



The heats of hydrogenation permit an estimation of the resonance energy of double-bond conjugation in the molecule. The expected value for the heat of hydrogenation if the double bonds did not interact would be twice the heat of hydrogenation of cyclopentene, i.e., 53.840 kg.-cal. per mole. The observed value is about 3.0 kg.-cal. per mole less. This can be ascribed to double-bond

interaction. It is equal to the difference between the second and first heats of hydrogenation in the stepwise reduction of cyclopentadiene to cyclopentane. This resonance energy is somewhat smaller than that usually associated with such conjugated systems.

D. OXIDATION

Cyclopentadiene reacts spontaneously with the oxygen of the air to form brown gummy products. Because the oxidation products may contain cyclopentadiene peroxide, they should be handled with great caution until more experience has been gained with their properties. Engler and Frankstein (80) found that, after a week's standing, 2 g. of cyclopentadiene had absorbed only 30 per cent of the oxygen theoretically required for formation of the diperoxide. They accounted for the low absorption by the formation of dicyclopentadiene and of a resinous coating which prevented contact between the oxygen and the inner layers of cyclopentadiene.

The properties of cyclopentadiene peroxides have received little attention. The results of some further studies made by Engler and Frankstein (80) on the oxidation products of dimethylfulvenes prepared by condensation of acetone and cyclopentadiene are suggestive of what might be expected. A 5-7 per cent solution of the fulvene in benzene treated with oxygen became turbid, owing to formation of an insoluble diperoxide. The accelerating effects produced by light and by an increase in temperature are presented as follows:

At 6°C. in the dark	Precipitation started after 21 days
At 6°C. in dispersed daylight..	Precipitation started after 14 days
At 19°C. in dispersed daylight	Precipitation started after 4 days
At 35°C. in dispersed daylight.	Precipitation started after 30 hr.
At 42°C. in dispersed daylight.	Precipitation started after 20 hr.

The primary product at the higher temperatures was the insoluble diperoxide, but a soluble form was a secondary product. The insoluble diperoxide exploded if heated to 130°C. or if ground with ether in a mortar.

The reaction of cyclopentadiene and oxygen was found by Stobbe and Dümhaupt (208) to be unaffected by light. Large amounts of gum were formed when a gasoline containing 10 per cent of cyclopentadiene was exposed to oxygen at 100°C. and 100 pounds pressure by Flood and coworkers (87). Gum formation was inhibited by the addition of 0.1 g. of α -naphthol per liter of oil.

In contact with catalysts cyclopentadiene has been almost completely oxidized. At 250-300°C. in the presence of manganese vanadate oxidation proceeded in 23 to 26 sec. with formation of carbon dioxide and a little carbon monoxide, according to Wilken-Jordan (243). The cyclopentadiene-air ratios varied from 1:0.86 to 1:1.10.

A 32.4 per cent yield of maleic acid was secured by Milas and Walsh (154) when cyclopentadiene was oxidized with air at 410°C. in contact with a vanadium pentoxide catalyst. Formaldehyde and carbon dioxide were identified in the

exit gases. Conover (57) patented the production of maleic acid by oxidizing cyclopentadiene with at least twenty times its weight of air at 400–525°C. in contact with a vanadium oxide catalyst. No advantage of cyclopentadiene over benzene for producing maleic anhydride has been indicated and the potential supply of the former is much smaller.

Cyclopentadiene reacted rapidly with perbenzoic acid to produce a dark brown, oily mass, according to Bauer and Bahr (33). Criegee (58) reported that lead tetraacetate oxidized cyclopentadiene with the production of 3-cyclopentene-1,2-diol diacetate and the monoacetate monoacetylglycolate of 3-cyclopentene.

E. POLYMERIZATION

1. *Formation of dicyclopentadiene*

Cyclopentadiene is readily polymerized and a variety of products can be obtained by the use of appropriate conditions. The spontaneous dimerization of two molecules of cyclopentadiene to form one of dicyclopentadiene, $C_{10}H_{12}$, has already been mentioned. The dimer occurs in the form of colorless crystals which melt at 32–32.5°C. and possess a camphor-like odor; the boiling point of the dimer is approximately 170°C.

Dicyclopentadiene was first identified by Roscoe (187) in 1886 in the products from the pyrolysis of phenol. He observed that the crystals decomposed to form a low-boiling compound when heated at atmospheric pressure, but distilled unchanged under vacuum.

2. *Rate of polymerization and depolymerization*

In their early work with cyclopentadiene Étard and Lambert (81) observed its spontaneous dimerization and measured the change in rate by the increase in density at intervals of time.

In experiments using the change in the refractive index as a measure of the dimerization rate, Stobbe and Reuss (209) found the temperature to be a vital factor. At –80°C. no polymerization occurred; at –15°C. a slow conversion was apparent; and at 20°C. cyclopentadiene was completely transformed to the dimer in 30 days. At 100°C. the reaction was still faster. Light had little effect on the rate of polymerization.

The rates of polymerization as measured by the refractive index were found by Terent'ev and Solokhin (217) to be in error by about 1.5 to 2.0 per cent, owing to the formation of higher polymers. This conclusion was reached by comparing the rates secured by refractive-index measurements with those obtained by a diazo method. The latter, which involved reaction of cyclopentadiene with *p*-nitrobenzenediazonium chloride in acetic acid, were followed by the change in color of the solution.

The magnetic susceptibility was used by Farquharson (86) to follow the rate of dimerization. The susceptibility of cyclopentadiene, -0.717×10^6 , decreased as the reaction proceeded.

Barrett and Burrage (32) prepared vapor pressure-composition curves from known mixtures of cyclopentadiene and its dimer, and used them to determine the rate of polymerization. After 93 days at 12°C. the mixture contained only 0.73 per cent of the monomer, and the transformation was apparently complete after a year.

The kinetics of the dimerization of cyclopentadiene in both the gaseous and the liquid states were extensively studied by Wassermann and his associates (32, 37, 82, 119, 125, 126, 127, 164, 235). In the gaseous state at constant volume the decrease in pressure was used to measure the rate at temperatures between 79° and 150°C. and pressures between 154 and 735 mm. The results were expressed by the equation

$$k = 1.2 \times 10^6 e^{\frac{-18.7}{RT}}$$

in liters per gram mole per second. The reaction was bimolecular and no indication of a chain mechanism was observed. Dissociation of the dicyclopentadiene into a monomer was a monomolecular reaction and in the pure liquid state the rate was expressed by the equation:

$$k = 6 \times 10^{12} e^{\frac{-34}{RT}} \text{ per second}$$

The parameters in the Arrhenius equation, $Ae^{\frac{-E}{RT}}$, for the association of cyclopentadiene when dissolved in both polar and non-polar solvents were similar to those for the reaction in the gas phase. The solvents included benzene, paraffin (125, 126), ethanol, acetic acid, carbon disulfide, and aniline (119). The solvent effects were not large enough to produce changes of A and E exceeding the experimental errors.

The rate constants obtained by Kistiakowsky and coworkers (104, 130) for the dimerization of cyclopentadiene and decomposition of the dicyclopentadiene were of the same order of magnitude as those calculated by Wassermann. The reaction rate in the gas phase was followed by the change in pressure, and in the liquid phase by the refractive index. In the latter case the rate started off as that of a second-order reaction and then changed to that of a first order, owing, it was concluded, to the formation of higher polymers.

Rate constants obtained by Schultze (196) for the dimerization of gaseous cyclopentadiene at temperatures between 132°C. and 182°C. and initial pressures between 180 and 510 mm. were similar to the values obtained by Wassermann.

Baur and Frater (34) followed the dimerization of cyclopentadiene and the dissociation of dicyclopentadiene at 149–194°C. and pressures of 109–638 mm. of mercury by measurements with glass-spiral manometers. The values of

$$K = \frac{(\text{cyclopentadiene})^2}{\text{dicyclopentadiene}}$$

were 277, 591, 742, 1177, and 2200 at 149°, 165.5°, 170°, 180°, and 195°C., respectively. At equal distances from equilibrium these investigators found de-

polymerization more rapid than polymerization. The heat of reaction was 12.3 kg.-cal. up to 185°C.; above that temperature polymerization was relatively more rapid. The energy of activation for polymerization was 5.4 kg.-cal.

The rate of dimerization was increased at 80°C. in the presence of oxygen, benzoyl peroxide, and diphenylethylene peroxide. Staudinger and Lautenschlager (206) attributed the increase to autooxidation and concluded that self-formed peroxides were more active catalysts than was benzoyl peroxide. No increase was observed at 45°C.

Hammick and Langrish (103) reported that the presence of peroxide is necessary for the dimerization of liquid cyclopentadiene in carbon tetrachloride solution at 25°C. Peroxides formed when the solution was exposed to the air for any length of time (according to the Kharasch ferrous thiocyanate test). Addition of 1 per cent of acetonitrile as an antioxidant reduced the rate of polymerization, and polymerization did not occur at all in acetonitrile solution. The rate of polymerization was calculated from bromine absorption tests which were run periodically on dilute solutions of the hydrocarbons in carbon tetrachloride under standardized conditions.

On the other hand, in the researches of Stobbe and Reuss (209), Schultze (195), Harkness and coworkers (104), and Benford and coworkers (37) on the rate of dimerization, this reaction was not influenced by the presence of oxygen peroxides even in relatively high concentrations. The various experiments were carried out between 14°C. and 177°C. and at pressures between 1 and 4000 atmospheres. The presence of acetonitrile did not affect the rate in the work of Benford and his associates (37). The conclusion was reached that peroxidic intermediates did not play a part in the reaction. Schultze (196) reported that gum formation in motor spirits proceeded in the absence of air. The presence of nitrogen or water did not affect polymerization.

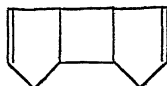
More information is obviously needed on the factors which affect dimerization; in particular, the differences in the effect of peroxides should be reconciled. The catalytic influence of other compounds, on which data are still meager, should be thoroughly explored. To be able to speed up the rate of dimerization would be especially advantageous in processes for the recovery of cyclopentadiene from oils.

Trichloroacetic acid was observed to be catalytically active in accelerating the rate of dimerization of cyclopentadiene in a benzene or a paraffin solution, and acetic acid was found to have an accelerating action in the gas phase, according to Wassermann (235a).

Ingold and Wassermann (116) found that the dimerization of cyclopentadiene in paraffin solution at 155°C. became heterogeneous in the presence of certain heavy-metal sulfides. The majority of the black sulfides were catalytically active, but the white and yellow ones were inefficient under the test conditions. The activity of a sulfide depended markedly on the method of preparation, possibly owing to the effect of impurities. They suggest that a periodic intermediate compound takes part in the reaction.

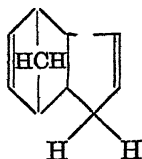
3. Structure of dicyclopentadiene

The structure of dicyclopentadiene was first represented by Kraemer and Spilker (140) with a cyclobutane ring between two cyclopentene rings:

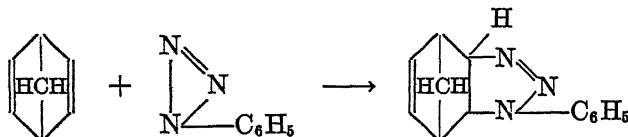


and for many years the work of other investigators supported this conclusion (203, 204, 207).

In 1933 Alder and Stein (15, 16, 20, 24) established that dicyclopentadiene had the structure



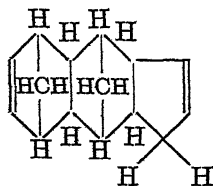
which corresponds to that of a partially hydrogenated indene containing a bridged methylene group. Thus, they found that dicyclopentadiene would add one molecule of phenyl azide, which is a characteristic reaction for bicycloheptene:



This reaction, however, would not occur with the double bond of a cyclopentene ring.

Pirsch (18, 174) supported the bicycloheptene structure with his work on the melting points of acenaphthene and a number of other aromatic compounds dissolved in dicyclopentadiene, dihydrodicyclopentadiene, and tetrahydrocyclopentadiene. The molecular depressions were high in all cases, averaging 46.2, 45.4, and 35, respectively.

The constitution of dicyclopentadiene indicates that polymerization has taken place by the Diels-Alder mechanism. The formation of higher polymers follows the same course; thus tricyclopentadiene has the structure:

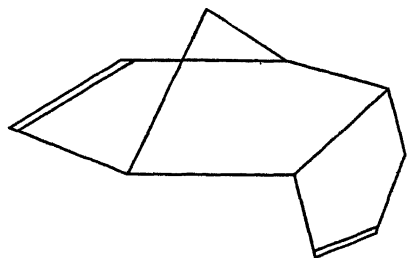


Each polymer is capable of adding another molecule in the same way. The formation of a continuous series of homologs is limited only by the physical

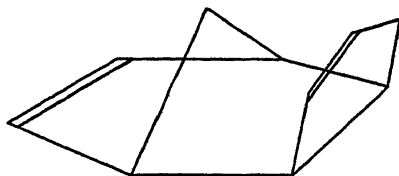
properties. The solubility of the higher polymers decreases so rapidly, however, that polymerization practically ceased with the formation of pentacyclopentadiene (20).

4. Stereoisomers

The polymers of cyclopentadiene with the bicycloheptene structure are three-dimensional. Two stereoisomers of dicyclopentadiene are possible:



Endo-form



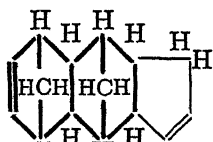
Exo-form

The former is produced, according to Alder and coworkers (20, 22), during polymerization at ordinary temperatures; the exo-form can probably be produced only at higher temperatures. When solid dicyclopentadiene was heated for several hours at 100°C., the product consisted of a mixture of the endo- and exo-forms. Derivatives of dicyclopentadiene possess corresponding endo and exo stereoisomeric forms.

Staudinger and Rheiner (207) separated endo- and exo-dicyclopentadienes by filtration of commercial dicyclopentadiene in a stream of carbon dioxide, followed by vacuum distillation. The distillate was crystallized and redistilled. The endodicyclopentadiene had a melting point of 32.5°C., but that of the exo-form was only 19.5°C. The boiling point of the latter was the higher of the two.

Waring and coworkers (232) obtained an isomer of dicyclopentadiene, which melted at 27.8°C., by allowing freshly distilled cyclopentadiene to stand at 15°C. After recrystallization, the melting point increased to 31.5°C. The refractive indices of both sets of crystals were the same,—1.5120 at 20°C. The lower-melting isomer had only a slight odor, but the dimer melting at 31.5°C. had the typical camphor-like odor.

Eight stereoisomeric forms are possible for tricyclopentadiene, but it occurs in only two forms. Alder and coworkers (18, 20, 24) have shown that the spatial configuration of that position of the carbon skeleton represented by thick lines in the formula



is exactly the same in each case, and that the isomerism is to be attributed to the alternative spatial arrangements that can be assumed by the cyclopentene ring with respect to the plane of the adjoining ring. It is probable that the two forms of the trimer arise by addition to the two known forms of the dimer, respectively.

Endo- and exo-tricyclopentadienes were separated by recrystallizing from alcohol. The endo-form, melting at 57–63°C., was filtered off. The studies of Alder and Stein (19) indicated that the endo-form had the higher energy content.

5. Formation of higher polymers

Higher polymers of cyclopentadiene were obtained by Staudinger and Rheiner (204, 207) by heating dicyclopentadiene in sealed tubes.

Time of heating, hours.....	14	22	90
Temperature, °C.....	150–60	170–80	200
Tricyclopentadiene.....	40	50	25
Tetracyclopentadiene.....	10	30	45
Pentacyclopentadiene.....	2	5	10
Unchanged.....	50	10	5

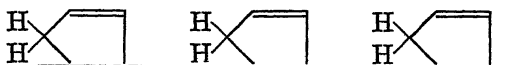
The trimer and tetramer were crystalline solids and gave normal values for the molecular weight by the freezing-point method in benzene. The pentamer was an amorphous solid which formed colloidal dispersions and gave abnormally high values for the molecular weight by the cryoscopic method.

These polymers were separated by fractional vacuum distillation and by selective solvents, and were identified by molecular-weight determinations. An insoluble solid was obtained which probably consisted of the hexamer and higher polymers. When heated to 500°C., again under low vacuum, the polycyclopentadiene depolymerized with the formation of cyclopentadiene (204). Kronstein (141) obtained polymers with similar properties by heating cyclopentadiene to 160°C. in sealed tubes for 5 hr. and dicyclopentadiene to 180°C. for 3 hr. Industrial applications for the wax-like polymers which can be produced by the above methods have not been described, but they appear deserving of attention.

Polymerization of cyclopentadiene in the liquid phase at temperatures of 0° to 40°C. and under pressures up to 5000 atmospheres proceeded in three stages, according to Raistrick and coworkers (178). The first was dimerization to endodicyclopentadiene, and then association to higher polymers occurred, but finally an explosive decomposition produced a highly carbonized residue, usually enclosed in a thin envelope of horny insoluble material. The gaseous products were 92 per cent methane and 8 per cent hydrogen. The decomposition was accompanied by a sudden pressure rise which amounted to 2000 atmospheres or more, depending on the experimental conditions and the volume of reactant used. The decomposition was very sensitive to such conditions as temperature, pressure, the diameter of the reaction tube, and the material from which the

tube was made. The explosive decomposition occurred within definite pressure limits at each temperature. Thus, at 13°C. it occurred at 6100 atmospheres; at 20°C., at 4900 atmospheres; at 30°C., at 3700 atmospheres; and at 40°C., at 3100 atmospheres.

Commercial resins have been produced by heat polymerization of cyclopentadiene and dicyclopentadiene without the use of catalysts. Carmody and coworkers (52) found that polymerization proceeded actively above 200°C. but that depolymerization might occur when the substances were heated above 260°C. A resin was produced from a close-cut coal-tar fraction containing dicyclopentadiene as the chief component by heating gradually to 200°C., but at no time to a temperature over 250°C., in the absence of air and catalysts (49). The product was of low molecular weight, heat-stable, color-stable below 225°C., resistant to oxygen and corrosive fumes, odorless, tasteless, and non-toxic. The average molecular weight indicated the presence of octamers, but a chain theory of polymerization,



was favored (52).

6. Polymerization with catalysts

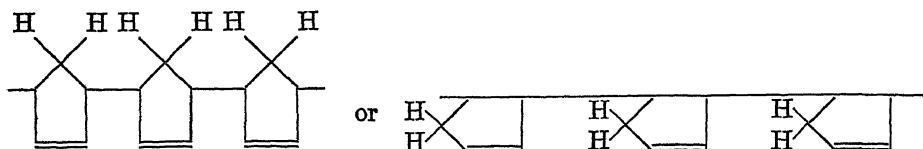
The influence of catalysts on the formation of higher polymers of cyclopentadiene has been studied by only a few investigators. Their results, however, fully justify a comprehensive investigation of the subject and examination of the products.

Brunson and Staudinger (44, 45, 205) produced high polymers by treating cyclopentadiene with many compounds, principally metallic halides. In many cases polymerization was rapid and proceeded at low temperatures. Thus, to 100 parts of cyclopentadiene dissolved in chloroform at a temperature of 0°C. or less was added 1 part of anhydrous stannic chloride dissolved in chloroform. Polymerization took place with evolution of heat; the solution became viscous and turned a deep orange color. After the solution had stood for 5 min., the polymer was precipitated as a white rubbery mass by the addition of an excess of absolute alcohol. The yield was quantitative. The stannic chloride combined with the alcohol to form a soluble compound which did not contaminate the rubber substance.

The polymer had the appearance of rubber. It could be vulcanized with sulfur chloride in chloroform and dispersed in rubber solvents, and was insoluble in ether, acetone, and ethanol. On standing in the air it absorbed oxygen and became insoluble in all known solvents, the final oxidation product having the formula $(C_5H_6O)_x$. The film formed by painting a benzene solution of the rubber on a surface became hard and insoluble on exposure to the air. The use of the polymer as a lacquer was suggested.

The polymers produced by Staudinger and Brunson (205) had the general formula $(C_5H_6)_x$, and molecular weights between 1260 and 6670. The polymers

added 1 mole equivalent of bromine per mole of cyclopentadiene. The following structures for the resin were suggested:

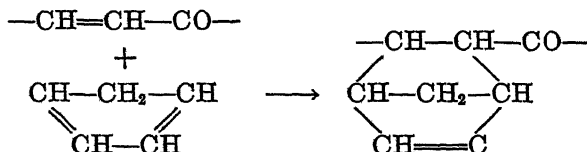


The use of phenyldiazonium fluoborate for polymerizing cyclopentadiene was patented by Bruson (43). Boron trifluoride, when mixed with another gas, such as nitrogen, and passed into cyclopentadiene produced a solid polymer, according to Chaney (53).

Soday (200a) and Trepp (223a) found that the cyclopentadiene polymers produced by certain metallic chloride catalysts were of two broad types, one of which is characterized by being soluble in solvents such as benzene, toluene, chloroform, carbon tetrachloride, and high-flash naphtha, while the other is insoluble in these solvents. The proportion of soluble polymer is influenced by four factors: temperature, concentration of cyclopentadiene, proportion of uniformly distributed catalyst, and time. The soluble polymer may be used for metal coatings. The catalysts were zinc chloride (200a), aluminum chloride (200a), ferric chloride (223a), aluminum bromide (223a), and titanium tetrachloride (223a).

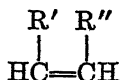
F. DIELS-ALDER REACTION

Cyclopentadiene can act as the diene in the Diels-Alder reaction with α,β -unsaturated carbonyl compounds. The products are cyclohexene derivatives having a bridged or endomethylene group:



The reaction is obviously similar to the dimerization of cyclopentadiene, except for the replacement of 1 mole of cyclopentadiene by an unsaturated carbonyl molecule.

Many Diels-Alder reactions of cyclopentadiene have been investigated by Diels, Alder, and their coworkers. The added reagents include maleic anhydride, maleic acid, acrolein, acrylic acid, itaconic anhydride, citraconic anhydride, quinones and other unsaturated compounds with the general formula

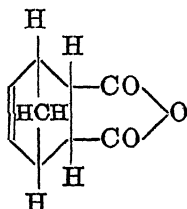


wherein R' and R'' stand not only for $-\text{CHO}$, $-\text{COOH}$, or $-\text{CO}-$ alkyl groups, but also for hydrogen or alkyl. In Norton's (167) thorough review of the Diels-Alder reaction these reactions have been included. The Diels-Alder reaction, therefore, presents a most fertile field for synthesizing from cyclopentadiene a wide range of compounds,—sesquiterpenes, alkaloids, camphors, and similar

hydrogenated cyclic compounds which are of interest to the resin, pharmaceutical, organic chemical, and other industries. McCrone (151a), for instance, has pointed out the structural similarity between pyrethrum and products obtained by the addition of cyclopentadiene to various unsaturated acids. He considers it possible that effective insecticides can be produced by the reaction.

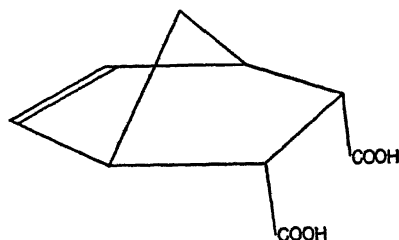
1. Maleic anhydride and derivatives

An important example is the reaction between cyclopentadiene and maleic anhydride to form *cis*-3,6-endomethylene- Δ^4 -tetrahydrophthalic anhydride (65, 69):

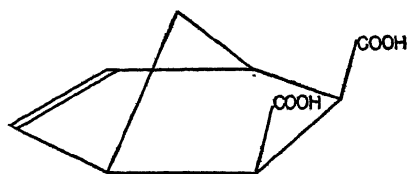


It occurs spontaneously with evolution of heat when cyclopentadiene is added to a suspension of maleic anhydride in benzene. The new anhydride is precipitated in the form of brilliant snow-white crystals, and the yield is quantitative. By catalytic hydrogenation or reduction with zinc and acetic acid the acid can be transformed into the fully hydrogenated system containing the methylene bridge (17). The endomethylene structure was established by Alder and Schneider (13). Thus, they obtained cyclopentane-1,2,3,4-tetracarboxylic acid by oxidation with alkaline permanganate.

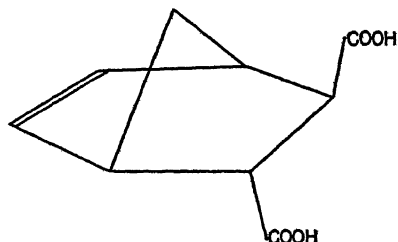
The 3,6-endomethylene- Δ^4 -tetrahydrophthalic acid occurs in the following three forms:



Endo-cis



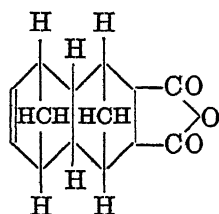
Exo-cis



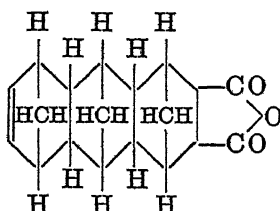
Only the *endo-cis*-form is produced from the reaction of cyclopentadiene and maleic anhydride (22). Alder and coworkers (23) produced the *trans*-form by the reaction of fumaryl chloride in ether with cyclopentadiene. The *trans*-acid has also been produced by heating the methyl ester of the *endo-cis*-acid with sodium in absolute alcohol. After removal of the alcohol and acidification the *trans*-acid was crystallized from solution (22). On distilling the *trans*-acid under vacuum some decomposition occurred; fumaric acid and the *endo-cis*-3,6-*endo*-methylene- Δ^4 -tetrahydrophthalic acid were found in the products. No *exo-cis*-acid was found (22).

The *endo-cis*-acid could not be converted to the *exo-cis*-form until the double bond in the dicycloheptene ring was saturated. When the dibromo*endo-cis*-acid was heated to 210–220°C., the dibromo*exo-cis*-anhydride was formed. The *exo-cis*-tetrahydrophthalic acid resulted on removal of the bromine with zinc dust and acetic acid (22).

Alder and coworkers (24) found that the double bond in the endomethylene anhydride was susceptible to further 1,4-additions with diene compounds. One or 2 moles of cyclopentadiene with 1 mole of the anhydride reacted to form



and



respectively. Similarly, butadiene, isoprene, diphenylbutadiene, or anthracene have been added to these compounds and to tetrahydrophthalic anhydride by Alder and his associates (24).

Morgan (158) has studied the reactions involving the dicarboxylic anhydride group of both the anhydride and the hydrogenated anhydride. The reactions were based on the corresponding reactions of phthalic anhydride, but in many respects the derivatives of the new anhydrides differed materially in behavior. The differences necessitated modifications in the modes of preparation and isolation of the derivatives. The investigation should be continued and the substitution of the endomethylenetetrahydrophthalic anhydride for phthalic anhydride in commercial applications for the latter should be tried.

Alder and coworkers (13, 21, 27) have described the preparation of lactones,

hydroxy acids, and other derivatives by treatment of the endomethylenetetrahydrophthalic acid with reagents such as diluted sulfuric acid or with oxidizing agents. By the addition of cyclopentadiene to maleic ester (27), maleic amide (110), dimethylmaleic anhydride (65), dibromomaleic anhydride (62), citraconic acid (61), or pyrocinchoninic acid anhydride (63), the corresponding derivatives of the endomethylenetetrahydrophthalic acid anhydride were produced.

The endomethylenetetrahydrophthalic anhydride can be used in place of phthalic anhydride for the production of alkyd resins, but nothing has been reported as yet on its use for this purpose. Hopff and Rapp (110) have made copolymers with styrene, acrylic ester, vinyl compounds, and other materials. The products varied from solids to rubber-like materials, depending on their composition.

Esters of endomethylenetetrahydrophthalic acid are high-boiling liquids. The methyl ester produced by the addition of cyclopentadiene to methyl maleate boils at 145–147°C. under 18 mm. pressure (22). The esters have been used by Jordan and Krzikalla (117) as plasticizers in nitrocellulose lacquers.

Diels and Friedrichson (71) showed that the same type of addition occurred when cyclopentadiene and acetylenedicarboxylic anhydride were mixed.

2. Additions with aldehydes, quinones, and acids

The formation of dicyclopentadiene is an example of a reversible association of the type $a + b = K_f/K_d$. Other examples are the addition reactions of cyclopentadiene with benzoquinone, acrolein, naphthaquinone, and other compounds.

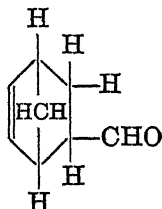
The equilibrium constant

$$K = \frac{K_f}{K_d} = B^{\frac{H}{T}}$$

has been measured and B and H evaluated for the additions of cyclopentadiene to acrolein, benzoquinone, and naphthaquinone, respectively, in both the gaseous state and in solution, by Benford and coworkers (36). The heat of reaction (H) was approximately 17 kg.-cal. per gram mole in these cases.

Rate constants for the reactions have been determined by Kistiakowsky and Lacher (129) and by Wassermann (124, 233, 235). The forward reaction was homogeneous and of the second order; the decomposition was of the first order.

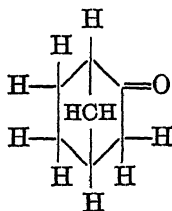
(a) *Aldehydes*: The compound produced by the addition reaction of cyclopentadiene with acrolein had the typical endomethylene structural formula:



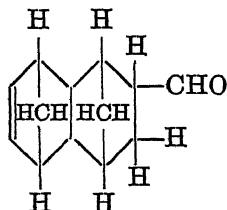
A 95 per cent yield of this endomethylene-3,6-tetrahydro- Δ^4 -benzaldehyde was obtained by Diels and Alder (65). Decomposition of the aldehyde into the

reactants was appreciable when heated to 150°C., and was rapid at 200°C., according to Kistiakowsky and Lacher (129).

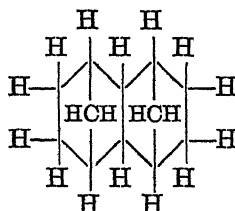
Compounds related to the terpenes have been synthesized from the addition products of cyclopentadiene and acrolein. Diels and coworkers (66, 68) hydrogenated the double bond in the endomethylenetetrahydrobenzaldehyde and eliminated the aldehyde group to produce norcamphor:



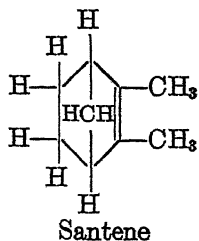
By addition of 2 moles of cyclopentadiene to 1 mole of acrolein, Alder and Windemuth (27) obtained the compound:



Subsequently, by reduction and elimination of the aldehyde group, they produced 1,4,5,8-bis(endomethylene)decalin:



Addition of cyclopentadiene and crotonaldehyde took place in the 1,4-manner when the two were heated at 100°C. for 4 hr. From the 2,5-endomethylene-6-methyl-Δ³-tetrahydrobenzaldehyde Diels and coworkers (68) prepared santene.

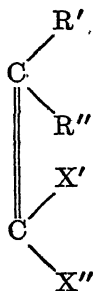


The addition products of cyclopentadiene and unsaturated aldehydes were used by Diels and Alder (64) to prepare products with flower-like odors. As condensing agents they used sodium or potassium methoxide or barium hydroxide.

(b) *Acids*: The addition product of acrylic acid and cyclopentadiene was obtained as an oil with an intense valerian odor (65). Komppa and Beckman (137) obtained the same product by the addition of cyclopentadiene to crotonic acid, and by heating dicyclopentadiene with crotonic acid to about 170°C.

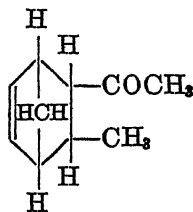
The addition of dimethylacrylic acid $(\text{CH}_3)_2\text{C}=\text{CHCOOH}$, to cyclopentadiene was observed by Komppa and Komppa (138) to proceed with considerable difficulty because of steric hindrance on the part of the methyl groups.

Alder and Rickert (11) added cyclopentadiene to a number of substituted, unsaturated malonic esters and to other derivatives of malonic acid having the general structure,



where R' and R'' included aliphatic groups, and X' and X'' included —COOR, —CN, or —COCH₃ groups.

(c) *Ketones*: Heating at 150°C. for 5 hr. was necessary for the reaction of ethylideneacetone and cyclopentadiene to form the compound

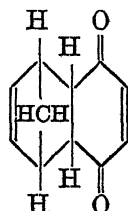


according to Diels and Alder (65). The reaction of cyclopentadiene with vinyl phenyl ketone, $\text{CH}_2=\text{CHCOC}_6\text{H}_5$, was studied by Allen and coworkers (29), and with the compound ($p\text{-ClC}_6\text{H}_4\text{COCH=}$)₂ by Adams and Wearn (2). When cyclopentadiene was added to *trans*-dibenzylethylene, *trans*-2,3-dibenzoyl-1,4-endomethylene-1,2,3,4-tetrahydrobenzene was produced.

The enols of β -diketo compounds contain double bonds in conjugation with a carbonyl group, i.e., an active double bond which should add to dienes. However, all such compounds investigated by Nylén and Olsen (168) were incapable of undergoing the diene synthesis. The following keto-enol compounds were

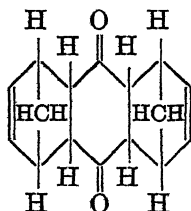
reacted with cyclopentadiene without results: acetoacetic ester (7.4 per cent), acetylacetone (76 per cent), benzoylacetone (94 per cent), dibenzoylmethane (96 per cent), ethyl benzoylacetate (29 per cent), ethyl oxalacetate (80 per cent), and oxalacetone. The percentages in parentheses represent the enol fraction in each case. To test the effect of the chelate structure on the above reactions, derivatives were chosen in which the hydrogen of the hydroxyl group was covered. Of the three investigated, only acetoxymaleic anhydride formed an addition product with cyclopentadiene. The investigators concluded that the oxygen at the active double bond did not in itself prevent the addition of the diene.

(d) *Quinones*: The reactions between cyclopentadiene and several quinones,—benzoquinone, α -naphthaquinone, and chloranil,—were described by Albrecht (5) in 1906. The additions are of the characteristic 1,4-type (36, 129, 235) and take place readily on mixing the reagents. Thus the compound



was produced from cyclopentadiene and benzoquinone.

When cyclopentadiene was added to cyclopentadienequinone, dicyclopentadienequinone



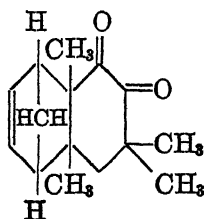
was formed (5), according to Diels and Alder (61).

The addition products of cyclopentadiene and quinones are highly colored compounds, and the changes in color of the solutions were used by Wassermann (233) to measure the rate of the additions. In benzene at 25°C., the rate constant for the formation of cyclopentadienequinone was about twenty-five times that for the dicyclopentadienequinone. Fairclough and Hinshelwood (83) found the formation of cyclopentadienequinone to be about five times as fast in polar as in non-polar solvents. The effects of catalysis on the rates of diene synthesis for cyclopentadiene with benzoquinone, α -naphthaquinone, and tetrachlorobenzoquinone have been studied by Wassermann (235a, 235b, 236). The experiments with sulfuric, phosphoric, and hydrochloric acids and with certain organic bases did not lead to conclusive results. Iodoacetic acid, *p*-toluenesulfonic acid,

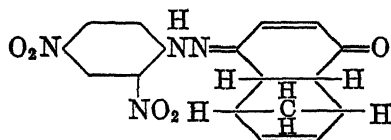
pyridine, 2,2'-dipyridyl, three salts of heavy metals, iodine, *trans*-stilbene, oxygen, peroxides, acetonitrile, and ethyl trichloroacetate were ineffective, but phenol, bromoacetic acid, chloroacetic acid, trichloroacetic acid, α -naphthaquinone, acetic acid, and trimethylamine were catalytically active. The last tests were made in both polar and non-polar solvents. An investigation of the kinetics of a selected list of catalytic processes showed that the primary step is of the third order and that the activation energy is near to zero. More information is needed on the effects of catalysts on the reactions.

In a study of the stereoisomeric forms of the quinones Alder and Stein (16) showed that the adduct of cyclopentadiene and benzoquinone can occur in an endo- and an exo-form. Although with two cyclic dienes attached to the quinones six forms are possible, only a single form resulted.

When Smith and Hac (199) added cyclopentadiene to tetramethyl-*o*-benzoquinone (prehnitenequinone), they obtained a 63 per cent yield of cyclopentadiene-prehnitenequinone:



Bergmann and Bergmann reported (38) that cyclopentadiene and *p*-phenylquinone will add. The addition reaction of cyclopentadiene and certain hydroxyazo compounds was used by Lauer and Miller (145) to show their tautomeric quinoid structure. Thus, with 2,3-dinitroazophenol the compound



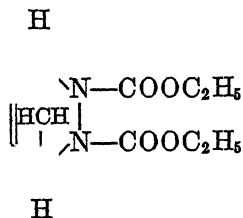
was produced.

No attempts to utilize the complex quinones in dyestuffs have been described, but they appear to merit some attention from this industry.

3. Reaction of cyanides and other nitrogen compounds

Cyclopentadiene has reacted with compounds in which the —CO— group has been replaced by the —CN group by means of the Diels-Alder method. By heating crotononitrile with cyclopentadiene under pressure 3,6-endomethylene- Δ^4 -tetrahydro-1-methyl-2-cyanobenzene has been prepared. A similar reaction with the nitrile of sorbic acid has been patented (112, 113).

1,4-Addition occurred when an azo ester and cyclopentadiene were combined. The cyclopentadieneazo ester,

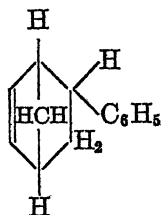


was hydrogenated to form endomethylenepiperidazine, according to Diels and coworkers (70).

4. Other Diels-Alder reactions

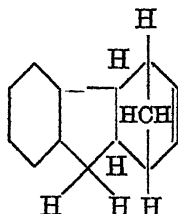
Cyclopentadiene and the methyl ester of acetylenedicarboxylic acid with cooling gave dimethyl 3,6-endomethylene-3,6-dihydro-*o*-phthalate. Diels and Alder (67) found that the ester decomposed into the original material on heating. By catalytic reduction the dimethyl ester of 3,6-endomethylene- Δ^1 -tetrahydro-*o*-phthalic acid was produced.

From styrene and cyclopentadiene the compound 2,5-endomethylene-1,2,5,6-tetrahydrodiphenyl,



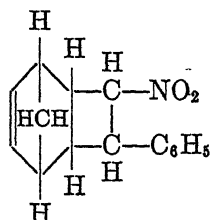
was formed. Alder and Rickert (9) noted that, like all bicycloheptene compounds, the product could be catalytically hydrogenated with unusual ease, to the hexahydro derivative. The double bond in the bridged ring underwent further diene syntheses with relative ease. The diphenyl, therefore, was always accompanied by a compound consisting of two molecules of cyclopentadiene and one of styrene.

The same investigators (10) found that fluorene derivatives could be produced by the addition of indene to cyclopentadiene. The product was largely 1,4-endomethylenetetrahydrofluorene,



together with a hydrocarbon in which two molecules of cyclopentadiene were combined, as in the case of styrene.

Cyclopentadiene and β -nitrostyrene reacted to give a 95 per cent yield of a compound which was apparently

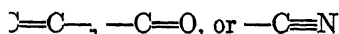


according to Allen and Bell (28).

The I. G. Farbenindustrie (115) has patented a process for the copolymerization of cyclopentadiene with styrene and other olefin compounds at temperatures below 0°C . in the presence of inorganic halides of an acid character, such as aluminum chloride, zinc chloride, or boron trifluoride. The polymerization was effected in the presence of an inert solvent, such as chloroform or carbon tetrachloride.

5. Reactions with nitro and sulfone compounds

In most of the previous additions of $\text{—}\overset{\text{H}}{\underset{\text{H}}{\text{C}}}=\text{CR}$ to cyclopentadiene, the R belonged to one of the groups



and conjugation of an unsaturated substituent to the double bond which was added was considered an essential prerequisite. Alder, Rickert, and Windemuth (12) have shown that this is by no means necessary. Cyclopentadiene has been

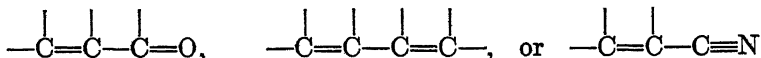
reacted with α,β -unsaturated nitro compounds, $\text{—}\overset{\text{H}}{\underset{\text{H}}{\text{C}}}=\text{CNO}_2$, and with sulfones,

$\text{—}\overset{\text{H}}{\underset{\text{H}}{\text{C}}}=\text{CHSO}_2\text{R}$, according to 1,4-addition. Nitroethylene and several of its homologs added cyclopentadiene easily at $100\text{--}110^{\circ}\text{C}$. On catalytic hydrogenation the adducts took up 1 mole of hydrogen with ease, and the resulting saturated nitro compounds were reduced to the corresponding amines with zinc dust and acetic acid. The 2-nitro-5-norbornylene obtained from nitroethylene and cyclopentadiene was sterically homogeneous. The adduct of cyclopentadiene and the cyclic sulfone butene-1-sulfone-1,4, $\text{CH}_2\text{CH}_2\text{CH}=\text{CHSO}_2$, resembled

dicyclopentadiene in external appearance and reacted very easily with phenyl azide.

6. *Reactions with vinyl compounds*

Vinyl compounds have been added to cyclopentadiene by reactions of the Diels-Alder type. The reactions are examples of a type of synthesis in which a double bond not belonging to the



types, and free from special constitutional influences of other kinds, proved capable of adding a diene. The reaction of vinyl compounds widens greatly the field for synthesis of new organic derivatives, particularly of the terpene family.

By heating cyclopentadiene and ethylene at 190–200°C. for 23 hr. at a maximum pressure of 5800 pounds, Joshel and Butz (118) produced bicyclo[2,2,1]-2-heptene

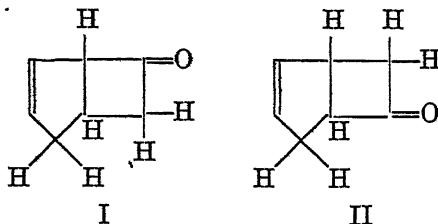


in almost quantitative yield.

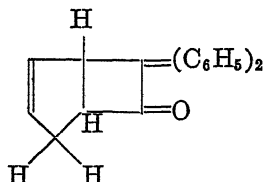
Alder and Rickert (8) obtained addition products by prolonged heating of cyclopentadiene and a number of vinyl compounds, including vinyl acetate, vinyl formate, vinyl chlorate, 1,2-dichloroethene, and trichloroethene. Alder and Windemuth (26) showed that addition with allyl compounds, including the alcohol, esters such as the salicylate or phthalate, the chloride, bromide, and iodide, and the amine, takes place smoothly at temperatures varying in the individual cases from 100°C. up to 180°C. Vinylacetic acid and vinylacetonitrile added more slowly than acrylic acid or acrylonitrile, but the reaction nevertheless took place at temperatures of 170–180°C. An adduct of cyclopentadiene and allyl isothiocyanate had an intense dill odor (26). Cyclopentadiene added Δ^2 -vinylcyclohexene to give the product 2,5-endomethylene- $\Delta^{3,3}$ -octahydro-diphenyl, according to Alder and Rickert (9).

G. ADDITIONS WITH KETENES

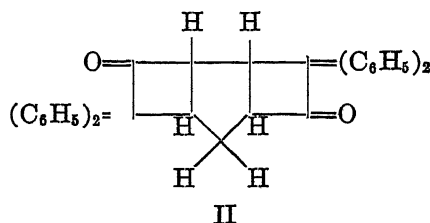
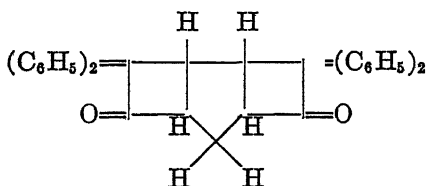
The reaction of cyclopentadiene with ketene to form an unsaturated ketone has been reported by Brooks and Wilbert (42). The product had either structure I or structure II.



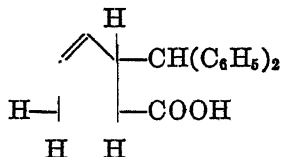
The reaction of diphenylketene and cyclopentadiene has been studied by several investigators. The structure of the product,



has been confirmed by the work of Smith and coworkers (198). Staudinger (201, 202) found that the white crystalline product could be decomposed into the original components by heating at 160–180°C. When the crystals were heated with more diphenylketene at 110°C. for 9 days, Smith and coworkers (198) were able to obtain a compound with structure I or structure II.



Farmer and Farooq (84) obtained an 85 per cent yield of the adduct of cyclopentadiene and diphenylketene. By alkali fission two isomeric forms of 2-benzohydrylcyclopentene-1-carboxylic acid,



were produced. Dimethylketene and cyclopentadiene have been combined by Lewis and coworkers (147).

H. OTHER POLYMERS OF CYCLOPENTADIENE

Synthetic resins which are copolymers of cyclopentadiene with indene, styrene, coumarone, and other unsaturated compounds have been produced from benzene forerunnings and polymerizable constituents of crude solvent naphtha or a polymer of these constituents. Carmody (50) agitated the mixture at a temperature of not over 20°C., while slowly adding aluminum chloride.

From coke-oven light-oil fractions which had been refined with sulfuric acid, Carmody and Kelly (51) recovered new aromatic compounds which they believe were formed by union of the unsaturated compounds, including cyclopentadiene, with the aromatics, such as benzene and toluene. The new compounds were high-boiling oils and were produced by steam distilling the residues from the distillation of the acid-refined light oil at 200–270°C.

The Friedel-Crafts reaction has been used for the preparation of resins from cyclopentadiene and acid chlorides, such as stearyl, lauryl, oleyl, or linoleyl chlorides. Acylation and polymerization of the cyclopentadiene proceeded simultaneously. The inventors, Ralston and coworkers (179, 180), used as a starting material crude solvent naphtha containing dicyclopentadiene as a major constituent, together with lesser quantities of coumarone and indene. The products of the reaction between the cyclopentadiene and the chloride were usually changed to hard resins by heating to 100°C. for a number of hours.

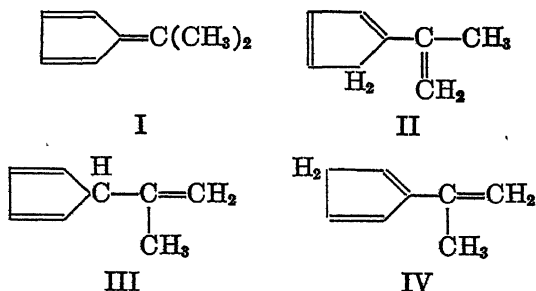
A composition resistant to corrosive influences was produced by mixing a dicyclopentadiene resin with a halogenated rubber and a styrene resin, according to Kodak, Ltd. (132).

I. CONDENSATIONS

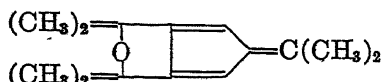
The methylene group ($-\text{CH}_2-$) is active, so that cyclopentadiene has been used in a number of condensations. This reaction also affords an excellent method for the preparation of a large number of new derivatives.

With aldehydes and ketones in the presence of alkaline condensing agents cyclopentadiene condenses easily to produce fulvenes in which the R and R' may be hydrogen, alkyl, or aryl groups. The color of the fulvenes increases with the size of the R and R', becoming blood-red with aromatic substitution. Condensations of cyclopentadiene with acetone, acetophenone, benzophenone, and other ketones were first carried out by Thiele (221). The colors of the products increased progressively from an intense orange to a blood-red. The aldehyde condensation products were also strongly colored, but resinified so easily that they could not be separated in the pure form. The condensation product from formaldehyde was a yellow oil of peculiar odor which changed very easily into yellow flakes, and absorbed oxygen rapidly.

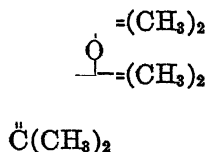
Ziegler and Crössman (247) pointed out the possibility that methylated fulvenes exist in several tautomeric forms, shown by structures I to IV.



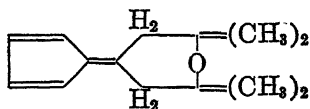
The methylene group in these fulvenes condensed further in certain instances. They suggested that the condensation product of dimethylfulvene with two molecules of acetone, which was prepared by Thiele and Balhorn (223), had the structure:



or

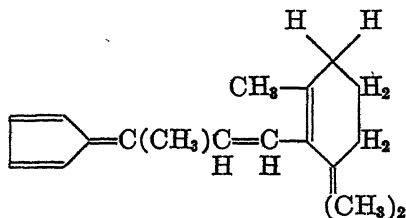


rather than



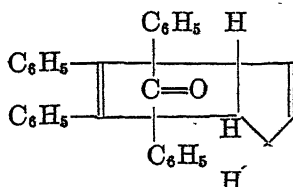
Kohler and Kable (134) condensed cyclopentadiene with cyclohexanone and cyclopentanone and isolated the corresponding fulvenes, which also were highly colored compounds and combined vigorously with maleic anhydride by the Diels-Alder reaction. The products were stable in the solid state, but when dissolved in indifferent solvents, such as ethyl acetate or benzene, dissociated into the original components. Dissociation was evidenced by the gradual disappearance of the fulvene color.

Basic fulvene derivatives were prepared by condensing cyclopentadiene with oxo-amino compounds or their *N*-monoallyl or *N,N*-dialkyl derivatives in the presence of an alkaline condensing agent. Diethylaminodimethylfulvene was produced, for instance, from $(\text{C}_2\text{H}_5)_2\text{NCH}_2\text{COCH}_3$ (4). An orange fulvene containing the β -ionone carbon ring,



was prepared by Willstaedt (244) by the condensation of β -ionone and cyclopentadiene. Kohler and Kable (133) obtained an 85 per cent yield of this product.

Tetraphenylcyclopentadienone and cyclopentadiene did not give the fulvene reaction. Grummitt and coworkers (101) refluxed the reagents in benzene solution for 4 hr. to produce a 75 per cent yield of 4,5,6,7-tetraphenyl-4,7-endocarboxyl-8,9-dihydroindene:

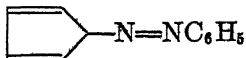


By hydrogenation of the fulvenes at 40°C. and 50 atmospheres pressure in contact with nickel on kieselguhr, Pines and Ipatieff (173) prepared alkylated cyclopentanes.

Reactions somewhat analogous to the formation of fulvenes have been reported by Thiele (221). Isonitrosocyclopentadiene, $C_5H_4=NOH$, was produced by the addition of ethyl nitrite, drop by drop, to a solution of cyclopentadiene in sodium ethylate and absolute ethanol. When ethyl nitrate was substituted for the nitrite, nitrocyclopentadiene, $C_5H_5NO_2$, was produced. The product was isolated as the sodium or silver salt, both of which were highly colored. With an oxalic ester the cyclopentadieneoxalic ester was produced. Both the nitro and oxalic ester compounds coupled with diazo compounds.

J. REACTIONS WITH DIAZONIUM COMPOUNDS

Cyclopentadiene and benzenediazonium chloride reacted in the presence of sodium acetate to produce brown crystals of cyclopentadieneazobenzene,



according to Eibner (75, 76). Guha and Hazra (102) could not induce cyclopentadiene to react with diazomethane in dry ether at 0°C., at room temperature, or in the presence of methanol as a catalyst, but found that cyclopentadiene would react with diazoacetic ester, $N_2CHCOOC_2H_5$, at 0°C. The product exploded when warmed to room temperature. Cyclopentadiene coupled easily with both diazotized *p*-nitroaniline and 2,3-dinitroaniline, according to Terent'ev and Gomborg (214). Thiele (221) reported that cyclopentadiene reacted with diazobenzene chloride in alcoholic solution to produce intensely colored compounds which resinified easily.

K. OTHER REACTIONS OF CYCLOPENTADIENE

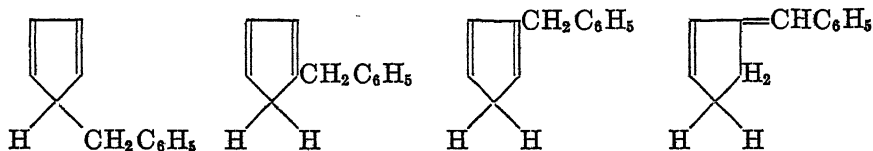
1. Cracking

When cyclopentadiene or dicyclopentadiene was passed through a tube heated to dull redness, a good yield of naphthalene, together with some benzene and anthracene, was obtained by Weger (237). Meyer and Hofmann (152) produced naphthalene by heating a coil of platinum wire to a dull red in an atmosphere of cyclopentadiene. In their opinion the five-membered rings were not converted to the hexagonal form directly, but were decomposed first to ethylene and acetylene. The latter then reacted to form naphthalene.

2. Reactions with metals and salts

Potassium reacted with cyclopentadiene in benzene solution to produce cyclopentadiene potassium, according to Thiele (222). Hydrogen was evolved and the reaction was strongly exothermic. Carbon dioxide was readily absorbed with production of the potassium salt of cyclopentadienecarbonic acid. By addition of dilute sulfuric acid the free acid was formed.

Cyclopentadiene potassium has been used by Alder and Holzrichter (6) to produce arylated derivatives of cyclopentadiene. By heating a mixture of the cyclopentadiene potassium with benzyl chloride, a mixture of 1- and 2-benzylcyclopentadienes and a little dibenzylcyclopentadiene were produced. The products were separated by vacuum distillation. Four isomeric benzylcyclopentadienes are possible:

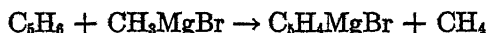


These derivatives formed dimers which could be decomposed, like dicyclopentadiene, by distillation at a controlled temperature. They all added maleic anhydride by the Diels-Alder reaction.

Gilliland and coworkers (94) were unable to obtain a reaction between cyclopentadiene and solid cuprous chloride, although other conjugated dienes, such as isoprene or butadiene, would react with the salt. Solutions containing cuprous chloride and ammonium chloride, however, were used for absorption of cyclopentadiene from gases by Luré and coworkers (149). Other dienes, such as piperylene, were also absorbed and a mixture of complex copper compounds was precipitated.

Mercuric chloride reacted with cyclopentadiene dissolved in methanol to produce a white precipitate which appeared to be a high-molecular-weight polymerization product, according to Hofmann and Seiler (109). The formula, $C_5H_4(HgCl)_2$, represents the proportions in which the reagents combined. The precipitate turned violet-brown in light and air.

Both halogen-substituted and addition derivatives of cyclopentadiene were produced by Grignard and Courtot (97) by use of the Grignard reaction. The reagent was prepared by the reaction of cyclopentadiene with the methyl Grignard reagent:



On addition of four atoms of bromine, tribromocyclopentene was formed. It would polymerize to the dimer, a brown powder. By decomposing the Grignard compound with cyanogen chloride the same investigators (97) obtained cyclopentadiene cyanide. After it had polymerized, the dimer was converted into dicyclopentadienedicarboxylic acid. Horclois (111) reported the production of cyclopentadienecarboxylic acid by treating the Grignard compound with carbon dioxide.

Cuprocyclopentadiene-triisopropylphosphine, $CuC_5H_5 \cdot 2C_3H_7P$ was produced by van Peski and van Melsen (171) by the reaction between cyclopentadiene, cuprous oxide, and triisopropylphosphine. It was suitable for use as an antiknock agent with gasoline.

3. Formation of alcohols

By reaction with hydrogen peroxide in contact with osmium tetroxide as a catalyst, cyclopentadiene in tertiary butyl alcohol solution was hydroxylated to 2-cyclopentene-1,4-diol, according to Milas and Maloney (153). By further oxidation under similar conditions, a 61 per cent yield of cyclopentane-1,2,3,4-tetrol was obtained. It was amorphous and extremely hygroscopic and turned black at 200°C. The alcohols from cyclopentadiene should prove interesting additions to this important family.

4. Reactions with sulfur and sulfur compounds

Cyclopentadiene readily reacts with sulfur at approximately 120°C. to form products which are at first slightly elastic, but become hard and brittle on standing, according to Mayberry (150).

Kruber and Schade (143) reported that the ethyl mercaptans and cyclopentadiene in motor benzol added to produce non-volatile resins. Posner (176) concluded that the mercaptans added at both the double bonds of cyclopentadiene. When cyclopentadiene and thiophenol were left in contact for several weeks and the product oxidized, a small amount of colorless crystals, which he identified as diphenylsulfonecyclopentane, $C_5H_8(SO_2C_6H_5)_2$, were obtained.

The formation of dyes or dye intermediates by condensation of a hydrocarbon containing the cyclopentadiene ring with a quaternary salt of a heterocyclic nitrogen compound containing as a reactive group either a *thioether* or a *seleno ether* group was patented by Kendall (122).

L. EFFECTS OF CYCLOPENTADIENE ON OILS

Cyclopentadiene or dicyclopentadiene in an oil will form gum; Schultze (196) concluded that gum formation by polymerization can occur in motor fuel even in the absence of air. The production of non-volatile resins in motor benzol by the addition of mercaptans to cyclopentadiene has already been mentioned (143).

Cyclopentadiene can be removed from an oil by washing with concentrated sulfuric acid. Claxton and Hoffert (56) concluded that cyclopentadiene and dicyclopentadiene were the substances chiefly responsible for the formation of sulfur dioxide during distillation of a coke-oven light oil which had been washed with acid. They found that the quantity of sulfur dioxide evolved was proportional to the amount of cyclopentadiene in the oil, and recommended that in the distillation process for removing the crude light oil from the wash oil the cyclopentadiene should be removed in a forerunnings fraction. Once dicyclopentadiene had formed, formation of the sulfur dioxide during the subsequent refining was very difficult to avoid. Sulfur dioxide formation could be minimized by using the lowest strength acid that would effect the required sulfur reduction, and by distilling at as low a temperature as possible. Dilution of the acid with a spacing agent, such as phosphoric acid, effected some improvement, but could not be recommended because of the cost. Boes (41) also considered cyclopentadiene responsible for sulfur dioxide formation during acid washing.

Hoffert and coworkers (107, 108) pointed out that in the presence of a high

concentration of unsaturated compounds, including cyclopentadiene, thiophene is removed from light oil with lower strengths and amounts of acids than would otherwise be the case. The loss of light oil will be correspondingly greater, owing to the concurrent destruction of the unsaturates by the acid. Kopelevitsch and coworkers (139) suggested the addition of a light oil containing cyclopentadiene to benzol before washing with acid in order to assist the removal of thiophene.

V. PHYSIOLOGICAL PROPERTIES

Only one study has been made of the toxicity of cyclopentadiene. Elfstrand (79, 229) reported that "the vapors cause narcosis of frogs in 10 minutes and it takes 70 minutes for the animals to recover. They show a primary motor unrest, the respiration becomes slow and intermittent and finally arrested. The animals finally develop flaccid paralysis and the reflexes are reduced, although not abolished. It causes a peculiar rigidity of the muscles which slowly disappears on recovery from the narcosis; with sufficient exposure, however, this may pass into rigor so that electric stimulation becomes ineffective. The rate and energy of the heartbeat are somewhat reduced and already at the beginning of narcosis cardiac peristalsis may set in. In rabbits the vapors already cause complete narcosis after 10 minutes, the respiration is markedly reduced, and the heart rate is increased; convulsions may develop during narcosis. The recovery is rather slow and only complete after 18 hours. In rabbits the subcutaneous injection of 0.5 to 1.0 cc. causes no narcosis, while 3 cc. causes narcosis with fatal convulsions. The material causes marked local irritation and autopsy exudates were found in the pleural and pericardial cavities, and the kidneys were hyperemic."

VI. IDENTIFICATION AND DETERMINATION

Cyclopentadiene has been detected in oils by means of color reactions. The green color produced in an oil containing cyclopentadiene by the addition of hydroquinone in methanol was used by Claxton and coworkers (56) as a qualitative test.

Vansheidt (227) found that solutions of hydrocarbons containing the group $(C=C)_2CH$, such as cyclopentadiene, indene, fluorene, and their derivatives, in acetone, pyridine, or naphthalene become colored on addition of alcoholic potassium hydroxide. On shaking in air the solutions are oxidized with a complete decolorization or color change. Vansheidt's reagent and *p*-dinitrobenzene produced characteristic green or blue colors with cyclopentadiene in acetone or pyridine, according to Levy and Campbell (146).

In his work on the reactions of aromatic diazo compounds with unsaturated compounds, Terent'ev (212, 215) found that by diazotization of *p*-diaminobenzene in phosphoric acid at $-10^{\circ}C$. with sodium nitrite in concentrated sulfuric acid, a reagent was obtained which gave a red to red-brown color with hydrocarbons containing double bonds. With cyclopentadiene the reaction was very sensitive.

Cyclopentadiene reacts mole for mole with *p*-nitrobenzenediazonium chloride.

By titrating the unreacted reagent with β -naphthol it was possible to determine the amount of the diene in a mixture (213, 219). The diazonium chloride also reacts with 1,3-diethylenic hydrocarbons, but not with aromatic hydrocarbons or compounds with conjugated hetero atoms. Terent'ev (217, 218) also stated that the reaction can be used for the determination of cyclopentadiene in a mixture with its polymer.

Two other qualitative reactions have been described by Terent'ev (216). When gas containing at least 0.25 mg. of cyclopentadiene was passed through an aqueous solution of mercuric nitrate slightly acidified with nitric acid, a white cloudiness appeared. Benzene, toluene, ethylene, divinyl, and isoprene did not interfere, but butenes and acetylene also caused precipitates, although less rapidly.

When 12–15 cc. of gas containing 0.05 per cent of cyclopentadiene was passed into a 0.25 per cent solution of *p*-benzoquinone in ethanol, a deep blue color developed. Even butene and acetylene did not interfere (216).

Afanas'ev (3) described another color reaction for cyclopentadiene. One drop was mixed with 1 cc. each of chloroform and glacial acetic acid and the product treated cautiously with two or three drops of concentrated sulfuric acid. As little as 0.1 mg. gave a distinct violet coloration. Terent'ev reported that the test was not specific for cyclopentadiene (216).

Cyclopentadiene gave an intense red color and a red precipitate with sodium nitroprusside in methanol solution made slightly basic with sodium or potassium hydroxide. Indene gave the same test, according to Scagliarini and Lucchi (192).

The Diels–Alder reaction of cyclopentadiene with maleic anhydride is a useful method both for its identification and for its determination (7, 61). Birch and Scott (40) used it for identification of diolefins in a compression gasoline produced in a high-temperature cracking unit. Other conjugated dienes, such as butadiene and isoprene, however, add with maleic anhydride by the Diels–Alder method.

The conjugated dienes in the fraction boiling between 20° and 80°C. from coal-gas light oil were determined by Gooderham (96) by the maleic anhydride reaction, in which the fraction is vaporized in a constant-volume steam-jacketed buret. Absorption of the cyclopentadiene by the maleic anhydride was complete in a few minutes.

Precipitation of the maleic anhydride adduct is quantitative. Dedusenko (59) separated the crystals by filtration and used them in determining the cyclopentadiene content of an oil.

Kirsanov and coworkers (128) treated freshly distilled cyclopentadiene with maleic anhydride for an hour. An iodide–iodate solution was added to the product and the excess iodine was titrated with thiosulfate. They reported that the method was accurate to ± 1 per cent. It was used for measuring the rate of dimerization of cyclopentadiene in a crude benzene. *p*-Benzoquinone could not be used for the addition with cyclopentadiene; after 3 hr. the reaction was only 85 per cent complete.

Cyclopentadiene and indene, Hammick and Langrish (103) found, can be

estimated with an accuracy of 2-3 per cent by determining the amount of bromine which reacts with a dilute solution of the hydrocarbon in carbon tetrachloride. A 100 per cent excess of bromine was used, and the concentration of the cyclopentadiene solution was less than 0.05 *M*. Under these conditions the cyclopentadiene added four bromine atoms and dicyclopentadiene added two bromine atoms. Schultze (195) also described bromination methods for the analysis of cyclopentadiene and dicyclopentadiene. The dienes absorb oxygen from the air so rapidly that quantitative bromination is impossible, but when titrated in vacuum or in the presence of an excess of free bromine from the very beginning of the test, the formation of peroxides is prevented and the results are quantitative.

VII. PROPERTIES OF DICYCLOPENTADIENE AND HIGHER POLYMERS OF CYCLOPENTADIENE

A. SOURCE AND PRODUCTION

Dicyclopentadiene is always found in cyclopentadiene when the latter is allowed to stand. The formation of the dimer from cyclopentadiene, and of the higher polymers, has been discussed at an earlier point in this article.

B. PHYSICAL PROPERTIES

The stereoisomeric forms of di- and tri-cyclopentadienes have been discussed above. Endodicyclopentadiene, the form produced by ordinary polymerization, occurs as colorless crystals with a camphor-like odor. The tri and tetra polymers are also crystalline, but the higher ones are amorphous insoluble compounds. Pentacyclopentadiene was reported to sublime at high vacuum by Staudinger and Bruson (204) and to separate as a gel from very dilute solutions. By heating the polymers were depolymerized to cyclopentadiene. Thus, cyclopentadiene split off when polycyclopentadiene was decomposed by heating to 370°C.

The physical constants for the polymers of cyclopentadiene which are available are tabulated in table 2.

The following values are available for the heats of combustion, fusion, and vaporization of endodicyclopentadiene and higher polymers.

COMPOUND	HEAT OF COMBUSTION AT CONSTANT PRESSURE (35)	HEAT OF FUSION (19)	HEAT OF VAPORIZATION (19)
	<i>kg.-cal. per mole</i>	<i>kg.-cal. per mole</i>	<i>kg.-cal. per mole</i>
Endodicyclopentadiene.....	1378.4	0.5	9.2
Endotricyclopentadiene.....	2055.8	5.1	11.1
Tetracyclopentadiene.....	2730.8		13.0

Alder and Stein (19) calculated the heat of fusion from cryoscopic data and the heat of vaporization according to Trouton's law.

Burchfield (46) has supplied the following constants for calculating the vapor

TABLE 2
Physical constants of cyclopentadiene polymers

POLYMER	MELTING POINT, °C.	BOILING POINT		DENSITY	INDEX OF REFRACTION
		°C.	Pressure in mm.		
Endodicyclopentadiene.	31.5 (232)	163	766 (19)	1.012 $d_4^{17.5}$ (solid) (187)	1.48031 $n_{H\alpha}^{77.35}$ (77)
	32 (19, 32, 204, 209)	170	760 (140, 202a)	0.9302 $d_4^{17.35}$ (77)	1.51047 $n_{H\alpha}^{20}$ (209)
	32.5 (240)	100	61.1 (140)	0.9756 d_4^{35} (140)	
	32.9 (187)	90	41.3 (140)	0.9766 d_4^{33} (140)	
	33.6 (131)	80	25.9 (140)	0.950 d_4^{37} (37)	
		70	24 (209)	0.942 d_4^{56} (37)	
		64-5	14 (135)	0.925 d_4^{78} (37)	
		61	14 (35)	0.888 d_4^{100} (37)	
		56	13 (240)		
		69	12 (204)		
Exodicyclopentadiene ..		51	0.1 (175)		
	19.5 (207)				
Tricyclopentadiene					
	68 (15)	254	766 (15)		
		110	3 (202a)		
	60 (202a, 204, 207)	105	3 (204)		
Tetracyclopentadiene ..					
	66 (24)	90-92	0.06 (207)		
	188-190 (202a)	344	766 (19)		
	190 (204)	160-5	1 (204)		
Pentacyclopentadiene ..		160	1 (202a)		
	270 (202a, 204)				
Hexacyclopentadiene...	373 (202a)				

pressure of dicyclopentadiene between 40.1° and 90.8°C. by the Clausius-Clapeyron equation: $A = 7.925$; $B = -2218$.

Tetracyclopentadiene and lower polymers are easily soluble in many organic liquids at ordinary temperatures, but pentacyclopentadiene is soluble in benzene and similar solvents only when hot. On cooling it is deposited as gelatinous needles. Polycyclopentadiene is completely insoluble (204).

The ignition point of dicyclopentadiene by the drop method was found by Zerbe and Eckert (246) to be 510°C. in oxygen and 680°C. in air. The antiknock properties of the dimer are good; the octane number was reported by Lovell and coworkers (148) to be over 100, the aniline equivalent 65, and the critical compression ratio 11.0. The addition of tetraethyllead lowered the critical compression ratio, Campbell and coworkers (48) found.

The dielectric constant decreased from 2.43 at 40°C. to 2.31 at 100°C., according to Waring and coworkers (232).

Infrared absorption spectra for dicyclopentadiene have been determined by Lambert and Lecomte (144). The differences in the spectra between dicyclopentadiene and its monomer have been pointed out previously. Kettering and Sleator (123) determined the infrared absorption in the region 1–30 μ for gaseous dicyclopentadiene by means of prisms of sodium and potassium halides, and plotted the per cent absorption against the wave length. The resolution was insufficient to separate rotational bands in any of the lines.

Raman bands have been tabulated for dicyclopentadiene by Reitz (106, 183, 185). The results of Lambert and Lecomte (144) indicate the greater complexity of the dimer structure over that of cyclopentadiene.

An x-ray investigation of polymerized cyclopentadiene by Hengstenberg (105) showed that although the tri-, tetra-, and poly-cyclopentadienes had different lattices, the molecules were all of the same size, 2.8Å., in one direction.

C. CHEMICAL PROPERTIES

1. *Reactions with sulfuric acid*

Although dicyclopentadiene is somewhat less reactive than cyclopentadiene, its properties and those of the higher polymers render these substances of interest in a number of industrial fields. The structure of dicyclopentadiene is rather suggestive. It is similar to that of indene, except that the former possesses a methylene group bridging the hexagonal ring, and two extra hydrogens. The indene structure renders dicyclopentadiene of some importance to resin producers. A large number of derivatives can be prepared from dicyclopentadiene, and useful applications for some of them are to be anticipated.

With dicyclopentadiene concentrated sulfuric acid reacts with charring, Boes (41) reported. In the presence of syrupy phosphoric acid or 50 per cent sulfuric acid dicyclopentadiene polymerizes without evolution of cyclopentadiene (235a, 235b). The resinous products produced with dilute sulfuric acid contain a sulfone, $C_{20}H_{22}SO_2$, a hard brittle mass, the amount of which depends on the strength of the acid. No appreciable amount of cyclopentadiene was evolved by

dry distillation of the sulfone. By the action of sulfuric acid diluted with acetic acid, deep blue derivatives have been obtained from poly- and di-cyclopentadienes (204).

2. Nitro compounds and reactions with nitric acid

The polymers of cyclopentadiene are oxidized by nitric acid, according to Staudinger and Bruson (204). The insoluble polycyclopentadiene is attacked with difficulty, but the lower polymers react more easily. Kraemer and Spilker (140) prepared the nitrosate by treating a solution of dicyclopentadiene in chloroform with nitric acid at -15°C . When dicyclopentadiene was mixed with amyl nitrite and acetic acid, the mixture cooled, and hydrochloric acid in 10 per cent alcoholic solution added, the nitrosochloride was produced. Dicyclopentadiene nitrosobromide was prepared by Rule (190) in similar manner. Wieland (239, 240) recommended the preparation of the nitrosochloride by heating dicyclopentadiene and bisnitrosochloride in amyl alcohol.

3. Halogen derivatives

Two molecules of a halogen will add to one of dicyclopentadiene, according to Kraemer and Spilker (140). Pauly and coworkers (169) found that dicyclopentadiene would add less than two atoms of iodine. The measurements of Ghosh and Bhattacharyya (92) indicated that the photoiodination reaction was unimolecular with respect to dicyclopentadiene.

Polycyclopentadiene did not decolorize bromine in a carbon disulfide solution, Staudinger and Bruson (204) observed. Tricyclopentadiene added 2 moles of bromine from a solution in carbon disulfide and tetracyclopentadiene added 1 mole. Pentacyclopentadiene decolorized the bromine solution. Deep blue, high-molecular-weight products, whose constitution has not been determined, were obtained by the action of bromine in acetic acid on dicyclopentadiene.

4. Hydrogenation

Dicyclopentadiene has been progressively hydrogenated through the dihydro to the tetrahydro derivative (17, 175). Nickel was used as the catalyst by Eijkman (78). The double bond in the bicycloheptene ring was the more reactive, and the heat of hydrogenation amounted to 33.2 kg.-cal. per gram mole. That for the pentagonal ring was 26.2 kg.-cal., according to Alder and Stein (19).

The higher polymers can also be hydrogenated. Alder and coworkers (24) dissolved tricyclopentadiene in methanol, added colloidal platinum, and agitated in an atmosphere of hydrogen. Data on the physical properties of the hydrogenated polymers of cyclopentadiene have been published by Alder and Stein (19) and Becker and Roth (69).

5. Oxidation

Dicyclopentadiene was oxidized easily by potassium permanganate in the cold, according to Kraemer and Spilker (140). In air dicyclopentadiene was resistant to oxidation below 300°C . and acted as an inhibitor for the combustion of more

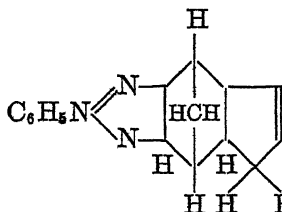
easily oxidizable hydrocarbons, such as decahydronaphthalene, Dumanois and coworkers (72) reported. Bauer and Bahr (33) found that when oxidized with perbenzoic acid dicyclopentadiene yielded a dioxide, which gave a mercuric chloride complex.

6. Reaction of sulfur and dicyclopentadiene

The reaction of dicyclopentadiene and sulfur has been studied over a wide range of concentration (150). At lower temperatures (below 105°C.) sulfur dissolved in dicyclopentadiene, the solubility ranging from about 1 per cent at 22°C. to 10 per cent at 100°C. Above 100°C. a reaction occurred with the evolution of hydrogen sulfide. This reaction increased in speed as the temperature was raised. Above 155°C. a vigorous exothermic reaction took place, accompanied by excessive foaming of the reaction mixture due to the rapid evolution of hydrogen sulfide. The characteristics of the products depended upon the composition of the mixture, the temperature, and the time of heating, and ranged from viscous oils to hard, brittle, non-crystalline solids. Mixtures containing up to 35 per cent of sulfur when heated less than 3 hr. below 155°C. yielded oils of varying viscosity. Plastic products were obtained when mixtures containing more than 50 per cent of sulfur were heated for 30 to 45 min. These products, although flexible and slightly elastic at first, slowly hardened and became brittle after standing for several days. Heating the reaction mixtures above 155°C. also produced hard brittle products. The solid products are only slightly soluble in most organic solvents. The resins may find application in the electrical industry or as a binder for the preparation of molding compositions.

7. Miscellaneous reactions

A characteristic reaction for the bicycloheptene ring structure is the coupling of phenyl azide, $C_6H_5N_3$, at the double bond in the endomethylene ring to give the addition compound (14):



The product is decomposed by mineral acids with liberation of nitrogen. Dihydrodicyclopentadiene will not react, according to Alder and Stein (15).

Komppa and Beckman (137) produced *trans*-3-methyl(exo)bicyclo[1,2,2]-heptene-2-carboxylic acid (endo) by the addition of crotonic acid to dicyclopentadiene. An iso-compound was also present in the products.

Wieland and Hasegawa (241) attempted to use the Friedel-Crafts reaction with acetyl chloride to synthesize unsaturated ketones. Dicyclopentadiene reacted vigorously but yielded no homogeneous product.

Mercuric chloride in methanol solution gave with dicyclopentadiene on 2 days' standing faint yellow needles of the compound $\text{ClHg} \cdot \text{C}_{10}\text{H}_{12}\text{OCH}_3$; in ethanol solution a similar compound, $\text{ClHg} \cdot \text{C}_{10}\text{H}_{12}\text{OC}_2\text{H}_5$, was produced, according to Hofmann and Seiler (109).

Sulfur chloride added to the polymers in chloroform solution, Staudinger and Bruson (204) reported, but the vulcanization product was impure and contained some acid.

8. Resin production

In addition to the previously mentioned dicyclopentadiene resins, the following have been described:

A coating for food containers has been prepared from solid polycyclopentadiene and styrene. Soday (200) copolymerized them by heating in a nitrogen atmosphere at 100–145°C. for 4–10 days. Methylstyrene yielded a similar product.

Dicyclopentadiene was one of the constituents in tar fractions which Rivkin and Sheehan (186) copolymerized with phenols to produce oily or resinous products; activated clay was used as a catalyst.

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HIGH-VACUUM SHORT-PATH DISTILLATION—A REVIEW¹

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I. INTRODUCTORY

This article traces the evolution of the short-path high-vacuum still with its growing technique and applications and attempts to offer a coherent story rather than a review of the whole field. The extended material is available in the bibliographies of Detwiler (23), in summaries by Burch (13), Fawcett (37), Burrows (16), Waterman (107), and again Detwiler (24), and in the rapidly expanding patent literature.²

The conditions for unobstructed distillation are simplicity itself, but they have led to an increasingly complicated and difficult practice. The process is no longer a new invention; it has become a self-contained technology, a school of

¹ Communication No. 54 from the Laboratories of Distillation Products, Inc. This article was completed in 1941, except for minor revisions.

² As we write, a compilation by D. D. Howat has appeared in *Chemical Age*, December, 1941-January, 1942.

thought. Three main branches have arisen, the Dutch under Waterman, the British inspired by Burch and Fawcett, and the American, first established at Kodak Park (52) and now transferred to Distillation Products, Inc. The European schools have summarized their technique; this paper stresses the methods of the Rochester group.

II. DEFINITIVE

Molecules are perpetually evaporating from liquids and solids. The number departing from the surface has been derived by Langmuir (80) from an equation introduced by Knudsen (77) which, in turn, was founded on the Maxwell-Boltzmann law:

$$n = \frac{ps}{\sqrt{2\pi MRT}} \quad (1)$$

when p , S , R , and T are the vapor pressure, the area, the gas constant, and the temperature, in appropriate units³. A square meter of platinum is unlikely to emit a molecule in many years. The same area of propyl phthalate loses about

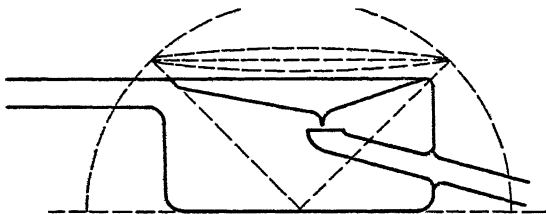


Fig. 1. Simple glass pot still showing cone of effective evaporation

2.5×10^{21} molecules or a cubic centimeter of liquid each second at 2 microns, which is approximately the vapor pressure of this substance at 25°C. Water ejects molecules so readily that the same relative quantity will evaporate even at the low temperature of dry ice. That water does not gasify spontaneously and propyl phthalate behaves as a fixed liquid is due to the surrounding atmosphere which knocks most of the molecules back into the surface. To secure active distillation, heat is used to increase the number and energy of evaporated molecules until they can drive away the obstructing gas and reach the condenser.

Distillation occurs at the lowest possible temperature when there is a complete absence of obstruction (mechanical obstacles or foreign gas), conditions typified by a drop of liquid evaporating freely into interstellar space. This ideal could be realized in the laboratory by placing a heated drop of liquid within a completely evacuated vessel with walls cooled sufficiently for every molecule striking them to condense. J. C. Hecker⁴ has simulated the arrangement by suspending the liquid in a spiral of electrically heated wire in an evacuated vessel. The liquid is held by surface tension and is free to evaporate in all directions not

³ Formulae in units useful to the chemist are given on page 75.

⁴ Patent applied for.

occupied by the wire. A simpler but less perfect interpretation of unobstructed evaporation is given by the laboratory pot still illustrated in figure 1. Liquid is heated in a shallow glass vessel connected with powerful vacuum pumps. Evaporation occurs without hindrance except by the sides of the vessel which prevent about half the molecules at any moment from condensing on the ceiling, whence they can be collected.

The process of free transfer under high vacuum from evaporator to condenser is known as *unobstructed-path* distillation; when the distance of transfer is comparable with the mean free path (75) of the vapor molecules in the residual gas, it is known as *molecular* distillation. The processes deserve attention because they are the simplest of all kinds of distillation; they occur at the lowest temperature and involve the least thermal decomposition. They make possible and therefore inevitable a revolution in the technology of one of the world's most plentiful raw materials,—the natural oils, fats, and waxes which hitherto have been considered undistillable.

III. HISTORICAL

The modern high-vacuum still has evolved gradually from the laboratory vacuum distillation train consisting of flask, condenser, receiver, manometer, and water ejector or mechanical pump. Chemists attempting to operate such apparatus at about 5 mm. of mercury found that further reduction of pressure in the receiver produced little increase in the rate of distillation or lowering in the readings of the thermometer placed in the neck of the flask (70). Minor variations in the design of the apparatus and in the supply of heat to the flask produced major alterations in the temperature and rate of distillation, a condition which resulted in elaborate instructions being issued to petroleum chemists for the standardization of distillation procedure. Substances of molecular weight above 300 could seldom be distilled, and a wide range of natural materials including heavy petroleum residues, vegetable and animal fats and their component fatty acids, and the natural waxes were all debarred from distillation. With improvement in vacuum technique it became appreciated that the bar to distillation was no longer the attainment of a sufficiently low pressure in the receiver but the resistance to the flow of vapor exerted by the neck and side arm of the distillation flask. The pressure of vapor in the flask was higher than the pressure in the receiver, causing decomposition and giving a false correlation between pressure and temperature, that is to say, the wrong boiling point. The state of affairs is illustrated in figure 2.

A quantitative key to the difficulties could have been found in the researches of M. Knudsen (76), who showed that the transfer of gases through tubes changes in kind and quantity at very low pressures. The transmission coefficient alters from one containing the viscosity of the gas and the fourth power of the diameter of the tubes, according to the laws of Poiseuille (84)

$$= f \left[\frac{D^4}{\eta L} \right] \cdot p(p_2 - p_1) \quad (2)$$

to one containing the third power and independent of viscosity.

$$= f \left[\frac{D^3}{L} \right] (p_2 - p) \quad (3)$$

The second equation is limited in its application by the double condition that the diameter of the tube must be small in comparison with the mean free path and with distances in which much change occurs in the density of the gas. There are end corrections which can be neglected only if the tubes are long. Although the volumetric transfer remains considerable, the weight of gases transported under high vacuum was found to decrease to extremely low levels. Thus, the newly introduced mechanical oil pumps would reduce the pressure to less than a

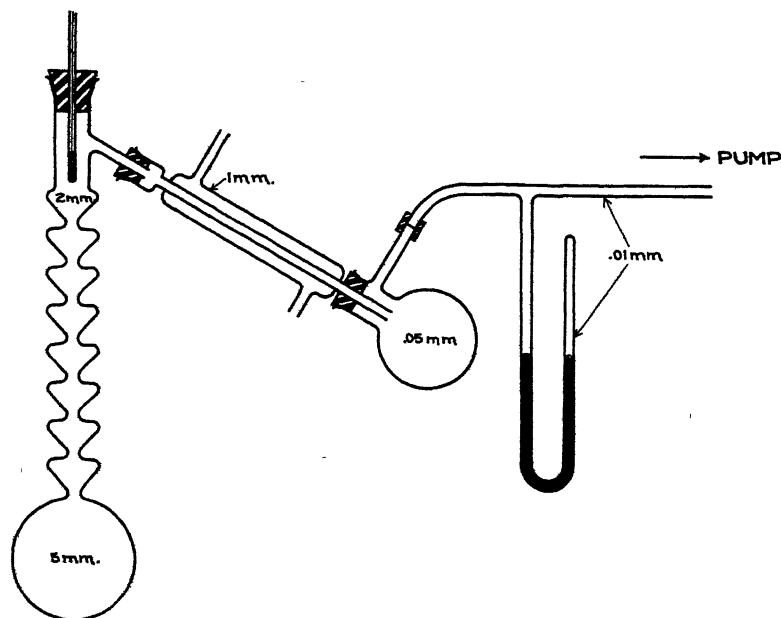


FIG. 2. Diagram of ordinary laboratory vacuum distillation apparatus showing probable pressures during distillation.

hundredth of a millimeter in the boiler of a laboratory vacuum distillation train, but the factor $(p_2 - p_1)$ in equation 2 representing the difference in pressure in the boiler and receiver became so small that the collection of distillate was prohibitively slow in apparatus of conventional design. Distillation done at these real pressures would take years and could be quickened only by (1) abandoning the low pressure and hence increasing the temperature and decomposition in the boiler, or (2) by redesigning the apparatus. It is this redesign of stills and the technique and consequences of their use which is the subject of this article.

The origins of open-path distillation stretch further back with each search of the literature. Detwiler's recent mimeographed abstracts (24) credit Krafft (79), Chevreul (21), and Caldwell and Hurtley (18) with being the first to harness

the x-ray vacuum to the laboratory still. Their methods remained unadopted by contemporary chemists, and it was not until the decade ending in 1920 that distillation equipment was designed with wide-necked flasks fitted with abnormally wide side arms (34) and much advantage was secured. (A recent version of the high-vacuum pot still (73) is shown in figure 3.) In the early 1920's German workers lowered into the distillation vessels condensers having trays (99)

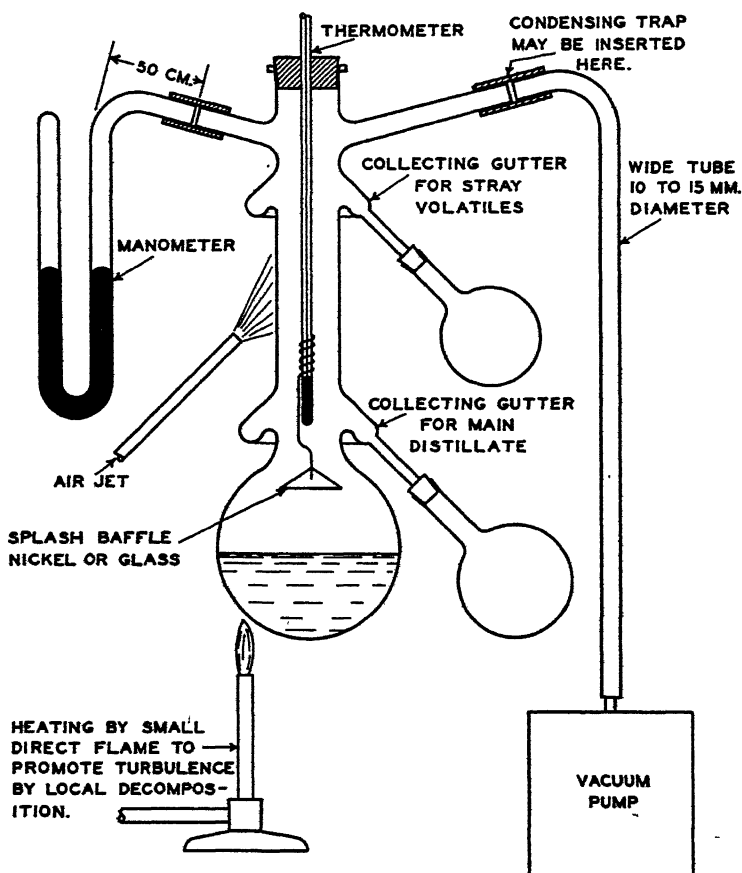


FIG. 3. High-vacuum pot still assembly

or umbrella-like lobes (103) to catch some distillate. The field was explored intensively by Waterman and coworkers (102) at Delft. About this time Brönsted and Hevesy, in order to separate the isotopes of mercury (10) by utilizing those distillation characteristics which depend on molecular weight as well as vapor pressure, produced a truly molecular still. A pool of mercury (figure 4) was allowed to evaporate slowly in high vacuum and condense upon the surface of a bulb cooled with liquid air and held just above the evaporating mercury. In the middle 1920's C. R. Burch (11) began experiments in high-

vacuum distillation. It is apparent that his experiments originated with an evaporative still and did not begin with study of conventional apparatus. Burch was one of the earliest if not the first worker to employ the Langmuir mercury condensation pump for producing the high vacuum in a still. Burch (12) examined the residue from petroleum refineries and demonstrated that he could distill a substantial proportion of this hitherto undistillable mixture. From vaseline he produced mobile liquid fractions of high molecular weights

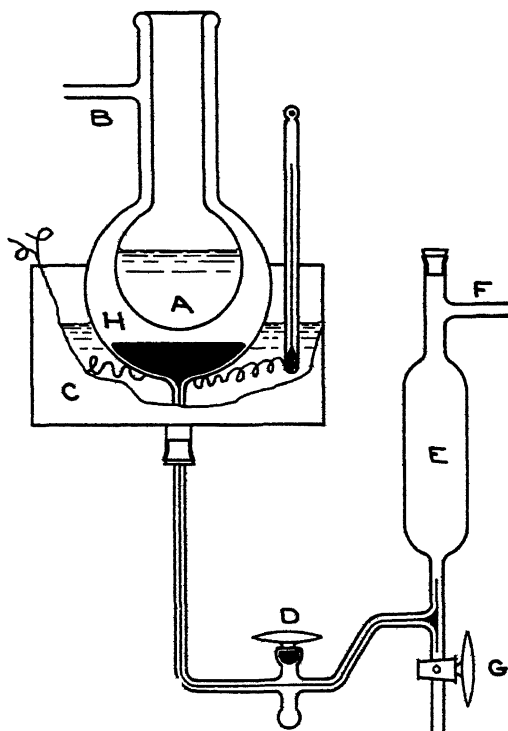


FIG. 4. Original molecular still used by Brönsted and Hevesy for the separation of the isotopes of mercury.

and exceedingly low vapor pressure. He introduced these as the first practical substitute for mercury to operate the Langmuir pump (81). In Langmuir's original patent (82) many liquids were mentioned for use in a diffusion pump, with mercury chosen as the most useful. Other inventors (72) suggested tin, potassium, sodium, etc., and even sulfuric acid as actuating liquids. Not only were Burch's hydrocarbons, now represented by the well-known Apiezon oils, the first serious substitute for mercury, but these oils and certain pure esters (5) devised for the same purpose have all but replaced mercury in high-vacuum pumps (51).

The molecular still of Burch's early experiments is shown in figure 5. An electrically heated tray was held within a glass vacuum-tight condenser which surrounded it entirely and was connected by a short wide tube to a condensation pump of diameter commensurate with the apparatus. In this simple assembly may be observed two of the cardinal points of a good molecular still: namely, that the condenser and evaporator shall be coembrasive, without intervening obstruction, and that the pump and admittance of the connecting tubes shall be large. It is interesting to note in many of the more complicated (108) and otherwise more efficient stills (25) built later that these requirements have not at first been met. The pumps and connecting manifold may well be as big as the still, a generalization which follows from Knudsen's measurements of gas flow in high vacuum (76, 84). Burch's experiments were translated into an industrial high-vacuum technology which was expounded in British (14) and later American (15) patents, where natural molecular paths were equated to the design and dimensions of the apparatus through the kinetic theory of gases. The data

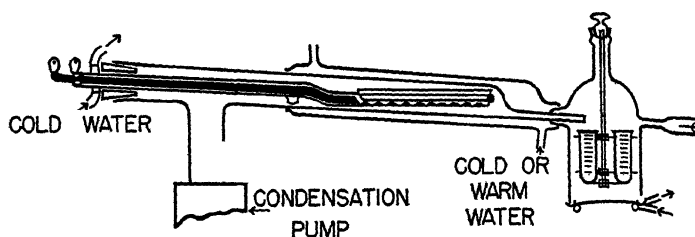


Fig. 5. Burch's flat-tray still first used to distill heavy petroleum residues

have indeed served as a textbook for later workers in the field. Burch's efforts were continued by a group of inventors (24), including Bancroft, Burrows, Fawcett, Sykes, Vigers and collaborators working in the laboratories of Metropolitan-Vickers Electrical Co., Ltd., and Imperial Chemical Industries, Limited. Whilst their endeavors were taking shape, Waterman with Elsbach, Oosterhof, van Vlodrop, and others at the University of Delft were progressing with the application of various pieces of high-vacuum equipment from the early distillation of coconut oil (105) to the study of drying oils (106). Although Waterman had at no time made the clear, sharp-cut statement of invention achieved by Burch, he and his school would appear to have done some of the earliest really high-vacuum distillation of natural oils (see, also, references 18, 21, and 79).

At about the time of these experiments a still was erected designed to produce some tons of heavy petroleum distillate daily (13), a development which was reflected in occasional short-path distillations done in American petroleum laboratories (109), although there is no record that the molecular still has reached commercial application in the petroleum industry either here or abroad. In America also three other groups were keenly interested in the high-vacuum

method. Carothers and Hill performed distillations and chemical reactions such as polymerization, with dehydration, in surface stills (19). Washburn at the Bureau of Standards made deductions of speed of distillation and degree of separation likely to be secured during the distillation of binary mixtures (101). The Kodak laboratories were the scene of experiments which had been started in 1928 on the distillation of plasticizers for motion picture film (49). The development of apparatus paralleled that of the German workers, beginning with low wide flasks (see figure 3) having vertical condensers and catchment rings, which were superseded by various kinds of pot stills (50, 69). A selection of pot stills designed by Kodak and other workers is given in figure 6.

In late 1930, a glass falling-film still was constructed in the author's laboratory and was used to repeat earlier experiments on the distillation of free vitamin D

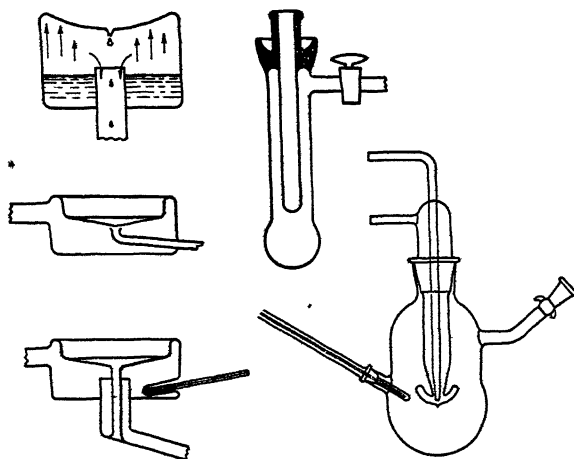


FIG. 6. Various glass pot stills

and esterified vitamin A from fish liver oils (59). Through the recommendation of M. H. Wodlinger the commercial possibilities of fish liver oil distillates came to interest General Mills, Inc., and in 1934 a joint project with the Eastman Kodak Company was launched to develop industrial short-path distillation. Concurrently in England, Carr and coworkers at British Drug Houses had commercialized the molecular purification of free vitamin A produced by the saponification of fish liver oils (20). A distillate secured by them in 1932 was by far the most potent preparation of vitamin A hitherto obtained and was then thought to represent the nearly pure product.

During the period from 1932–1936 Waterman (89, 104), and Fawcett *et al.* (41) severally explored the production of stand oils by the distillation of polymerized oils of high iodine value to remove the unpolymerized portion. The many important advances which resulted from this work have been abstracted by Detwiler (24), to whom the reader is referred. The work has been expanded by R. S. Morse (88) and successors at Distillation Products, Inc. Fawcett (36)

found that while most natural oils can be distilled in crude form, they are handled more readily if given a preliminary wet treatment to precipitate the proteins, phospholipides, glucosides, etc., which otherwise mar the hot columns.

Although the technology of short-path distillation was still far from complete, the question by 1935 had become not so much "how" as "what" to distill. The answer then was, "Valuable materials that can carry the cost of a partially developed process, extravagant in the use of apparatus and power." This criterion until recently has disposed of ventures into the petroleum and paint oil fields and left the concentration of the oil-soluble vitamins as the chief patron of the new art.

IV. EVOLUTION AND DESIGN OF THE HIGH-VACUUM SURFACE STILL

The short-path distillation of a constituent from a mixture occurs in four stages: (1) travel of the desired component from the body of the liquid to the surface; (2) distillation from the surface; (3) travel across the vapor gap; (4) condensation. Stages 2 and 3, which will be examined later, are readily facilitated by the proper temperature and vacuum, and stage 4 may be neglected because with substances which wet the condenser and with temperature differences contemplated in this article condensation is virtually complete. Stage 1, arrival at the surface, can become a limiting factor to such an extent that the design of the vacuum surface-still follows chiefly the requirements of the distilland layer.

Renewal of the surface occurs fairly rapidly under ebulliative distillation at normal pressures so that it is sometimes unnecessary to consider this factor in design. As the pressure falls and the molecular weight and viscosity of the distilland increase and as ebulliative distillation gives place to quiet surface evaporation, the bringing of the desired component to the surface becomes a major concern. A function of the highly efficient columns of the Podbielniak type is to delay exit of the vapors until all the chosen constituent has been evolved from the distilland. Under certain conditions this function is as important as sorting out the vapors after evaporation. In the evaporative still, surface renewal is at its slowest and there is no ready means for delaying the evaporation of unwanted constituents. It is imperative to keep the layer of distilland as thin as possible.

There is a second reason for a shallow distilland—decomposition. Thermal destruction can follow a number of courses, one of which terminates in explosion. More usually decomposition is a smooth function of temperature over a wide range, so that the loss of material is determined by temperature and the time of exposure. Therefore, although the establishment of molecular conditions surrounding the distilland may have reduced the *rate* of thermal destruction to an irreducible minimum, there remains almost unlimited scope for reducing actual destruction by diminishing the time of exposure. The need to renew the surface and the need to reduce decomposition both demand a flash-boiler which can operate with the thinnest film of distilland.

The first attempts to use thinner layers and better stirring than was feasible with pot stills were made by Burch and Bancroft (14), who allowed the distil-

land to wander through a series of trays, as in figure 7, supported within a cooled and evacuated cylinder. The evaporation from some of the trays was used to assist vacuum production and thus promote evaporation from other of the trays in a "self-pumping" still (see page 78). That this multiple arrangement gave better separations than a single tray showed that stirring had become an important factor.

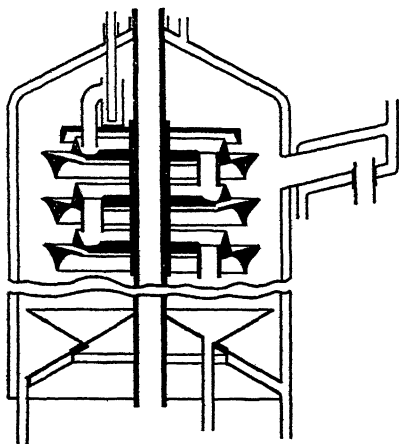


FIG. 7. Cascade tray still of Burch and Bancroft

A. The falling-film still

The next development was the falling-film still. The distilland is allowed to flow by gravity down a hot vertical surface on which it spreads in a layer generally 0.1–1.0 mm. thick according to viscosity and rate of feed. In figure 8 is shown the apparatus first used for separating vitamins A and D in the Kodak laboratory. This still is typical both as to virtues and faults of others (25, 104) described about this time. Since with minor alterations, such as increase in size of pumps and vacuum ducts, it embodies everything necessary for efficient laboratory distillation, a paragraph may be devoted to its description. The material to be distilled is admitted from reservoir A through a flow meter, sight glass, dropping pipet or other device, B, to a preheating tube, C, whence it enters the "explosion bulb," D, which bulb may be attached to the laboratory water pump or preferably to a single-stage mechanical pump at E. In the latter case a dry-ice trap may be interposed in the line. This is convenient when the preparation contains traces of solvents remaining from another operation. It is possible to allow the oil to enter the still directly, but it is preferable to provide another stage of degassing, F, which may be connected with the vacuum line servicing the condensation pump, G, attached to the main part of the still. The still itself consists of a central heated column, H, surrounded by a transparent condenser, K. The spent distilland flows into the reservoir, L, and the distillate into the receiver, M.

Much has been written concerning the best surface for spreading the distilland. Where moderate rates of flow—e.g., 0.15 cc. per square decimeter per second—are involved, glass or polished chromium-plated nickel is believed to be superior. With more rapid streams a spiral or knurled embossed pattern (56) helps to prevent the oil breaking into rivulets. Where the flow is extremely slow, a tenth to a hundredth of the amount mentioned above, a finely matted surface is favored by Detwiler (25) and coworkers. In the present case a spirally

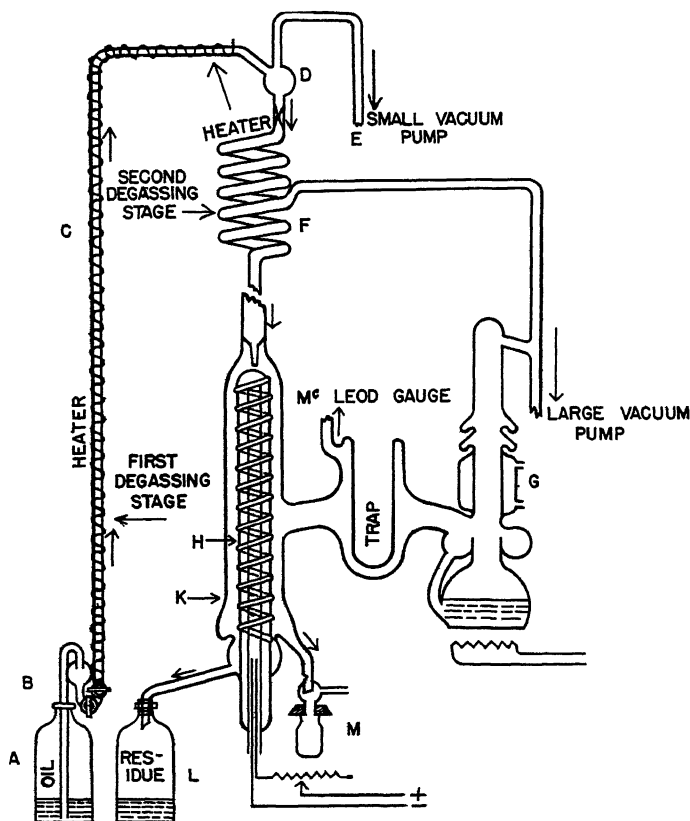


FIG. 8. Glass falling-film still first used to distill vitamins from fish liver oil

embossed glass column is shown. Admittedly much of the oil runs in a thicker stream around the spiral instead of flowing evenly over the surface. This tendency has been used to advantage in industrial apparatus by E. H. Payne (90). The temperature of the glass still can be maintained constant by using the vapor of a liquid of suitable boiling point, such as aniline or ethyl lactate. Liquids of considerably higher boiling point can be employed at lower pressures with a manostat which allows temperature control with some exactness (25). Another device is the electrically heated column where resistance winding replaces the heating fluid. The disadvantage of the electrical method is that the

temperature varies inversely with accidental variation of flow of distilland, whereas to maintain the distillation ratio it should vary superproportionately with the flow.

The industrial interpretation of the falling-film still has varied according as to whether the design is in the hands of chemists or of engineers. The chemist, wishing to obtain optimum conditions for the materials under treatment, has insisted on transparent apparatus in which the distribution on the column, the degree of separation, the amount of carbonization, etc. could be under constant surveillance. This has meant apparatus of low floor-space efficiency. The engineers have demanded all-metal construction with a grouping together of more than one distillation unit within a container. Burch and Bancroft's commercial stills for petroleum contained many flat trays in cascade or five or more cylindrical evaporators arranged side by side on a base-plate covered by a single removable tank. The flat-plate still (97) described by Burrows (16) used thick slabs of aluminum through which hot oil could be circulated to provide the heat for distillation. The distilland was distributed at the top of each slab and caused to flow down either side as evenly as possible, and a collection of N heated plates was sandwiched between $N + 1$ condensers within a vacuum tank. The advantages of such a compact design need no stressing; the disadvantages are lack of visibility and difficulty in cleaning. It is possible that this interleaved construction may find further application in the centrifugal still where many plates, mounted on one shaft (64), can be cleaned by their own motion during operation.

The stills which have survived in the author's laboratory are of a type that can be inspected continuously and dismantled when a defect develops, to be cleaned in the shortest possible time and put back into service. Our falling-film stills (52) have consisted, therefore, of little else than a series of vertical tubes standing on hollow metal bases which serve as connectors for the vacuum pumps, the columns being covered by bell jars as shown in figure 9. Cooling has been by air blast and heating by radiant heaters first introduced, we believe, by Fawcett. While this heat supply gives an admirably uniform allowance to each element of distilland when the latter is properly distributed, it places the entire assembly in unstable mechanical equilibrium. Let the oil "channel" ever so slightly, then the side receiving less oil becomes hotter and causes the column to expand vertically on this drier side, tipping the top of the column over to the wet side and aggravating the condition. The condition can, of course, be corrected with special alloys or compensating devices, but cost is an item.

Many schemes have been devised for distributing the oil evenly at the top of flat-plate and column stills. One group has favored a cascade divider (4) and another a dam of V-shaped serrations (52), but of all methods yet tried a rotating distributor appears to be the best (66). The oil having been distributed, it is necessary to prevent channelling during subsequent flow down the column. We have placed our faith in using designs embossed on the columns and placing a number of columns in series or series-parallel arrangement to redistribute the

distilland. Fawcett (39) has suggested redistribution from gutters placed at intervals around the column, a scheme which deserves more serious consideration.



FIG. 9. Industrial falling-film still. Glass dome on the left covers the fifth evaporator in a succession of individual stills. The panels for controlling temperature and pressure are shown on the right.

B. The centrifugal still

From the rotary distributor it is a short step to the design of a completely rotating still (63). Here the inherently simple act of molecular distillation, itself the simplest variety of evaporation, reaches its simplest expression; a

frying pan is twirled in a completely empty chamber. We constructed a short-path centrifugal still in 1935 in the form of a deep cone spun from sheet aluminum. It was followed by a still shown in figure 10 (with condenser removed), having a stainless-steel rotor 7 in. in diameter turned to form a shallow cone with a deeper depression in the center. The plate was surrounded by a gutter to collect the oil which flew from the edge in a fine spray. The gutter being water-cooled, the distilland was maintained at the distillation temperature for a

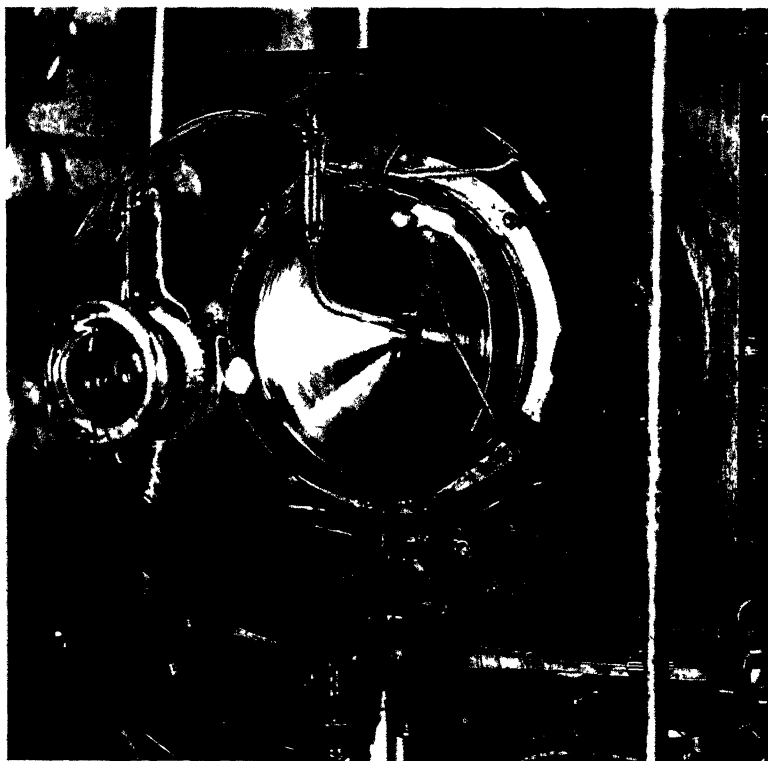


FIG. 10. Early centrifugal plate still. The oil is fed through the glass tube to the center of the plate whence it travels outwards towards the stationary, cooled collecting gutter. The condenser has been removed to give a better view of the evaporator.

small fraction of a second in a layer which was considerably thinner than had been secured with a falling-film still. The layer remained uniform in structure and the distilland advanced uniformly from center to the edge under trying conditions of temperature and variations of surface tension. A diagram showing the essential parts of a centrifugal still is given in figure 11. It was soon found that the cooled stationary gutter could be replaced by a rotating gutter and pick-up (61). Here the frying pan must be abandoned for a gramophone analogy, oil being fed to the center of the record and picked up at the periphery, an arrangement which is shown in figure 12. The distribution of charred material on

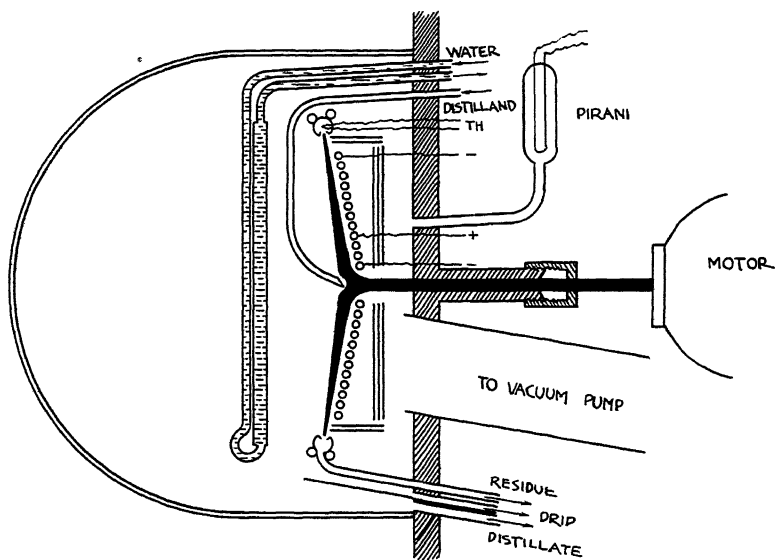


FIG. 11. Diagrammatic vertical section of centrifugal still

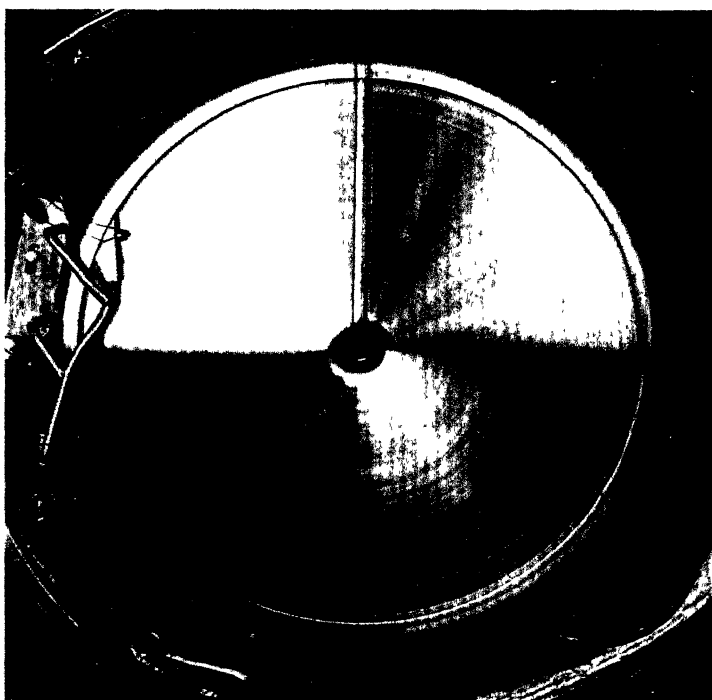


FIG. 12. Centrifugal plate with rotating collecting gutter and oil "pickup"

a still which has been allowed to become dirtier than usual is shown in figure 13, which may be compared with a similar condition on the falling-film column in figure 14.

While it can not be said that the centrifugal still has entirely displaced the falling-film variety, it holds first interest at the moment. Perhaps the future will call for a cross between a centrifugal and a falling-film still in which the more

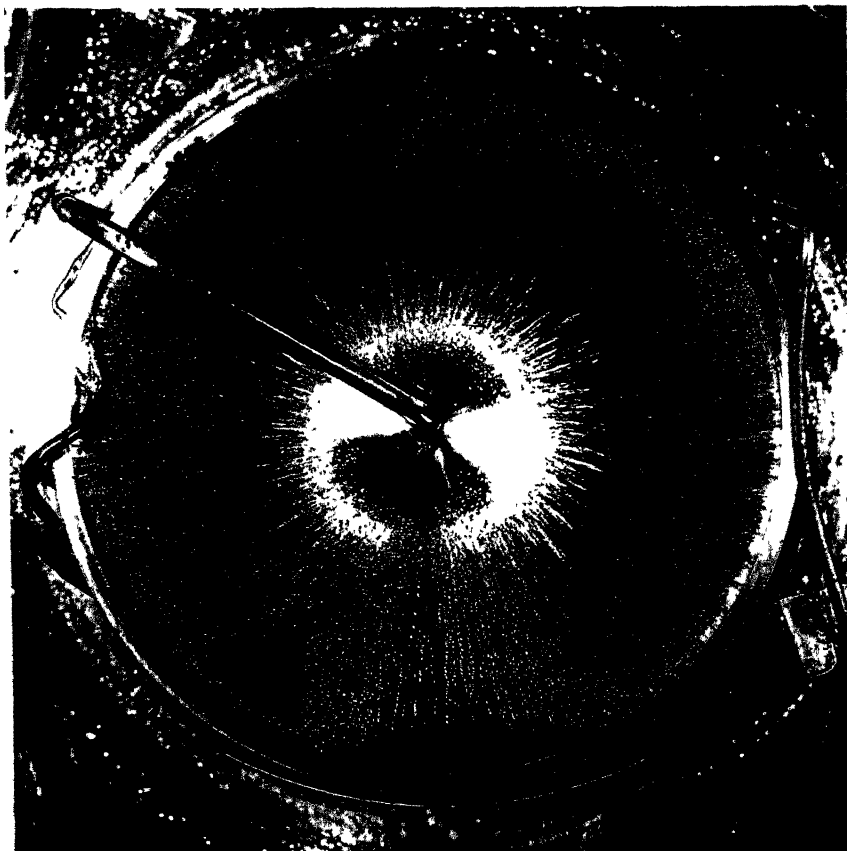


FIG. 13. Small centrifugal distillation plate after one week's continuous operation showing uniform distribution of charred deposit.

delicate constituents of an oil are removed on the rotating evaporator, which then distributes the stripped distilland to a stationary column for distillation of the glycerides. Although the temperature on the stationary column will be higher and the film thicker, the stripped glycerides can sometimes withstand this rougher treatment. Information concerning the early forms which multiplate centrifugal stills have taken industrially can be gleaned from figures 15 and 16, which depict batteries of small vertical plate and larger horizontal plate units used for the distillation of naturally occurring vitamins A and E.

There are some research aspects of the centrifugal still to which we may devote attention. At present the rate of spinning varies from a few hundred up to about 5000 R.P.M. There is little inherent reason why speeds approaching that of the ultracentrifuge should not be employed. Then if the liquid is ap-



FIG. 14. Falling-film still showing unsymmetrical deposition of deposit after two days' operation.

plied to the center of a plate or cone, only the narrow periphery of which is heated to distillation temperature, travel of a thousandth of a second or less across the heated portions may be expected and it should be possible to distill, without harm, materials of very high molecular weight.

The centrifugal still and especially the rim type provide a better separation

than is possible from simple molecular distillation. The mixture of distillate molecules will leave the surface with average speeds proportioned according to

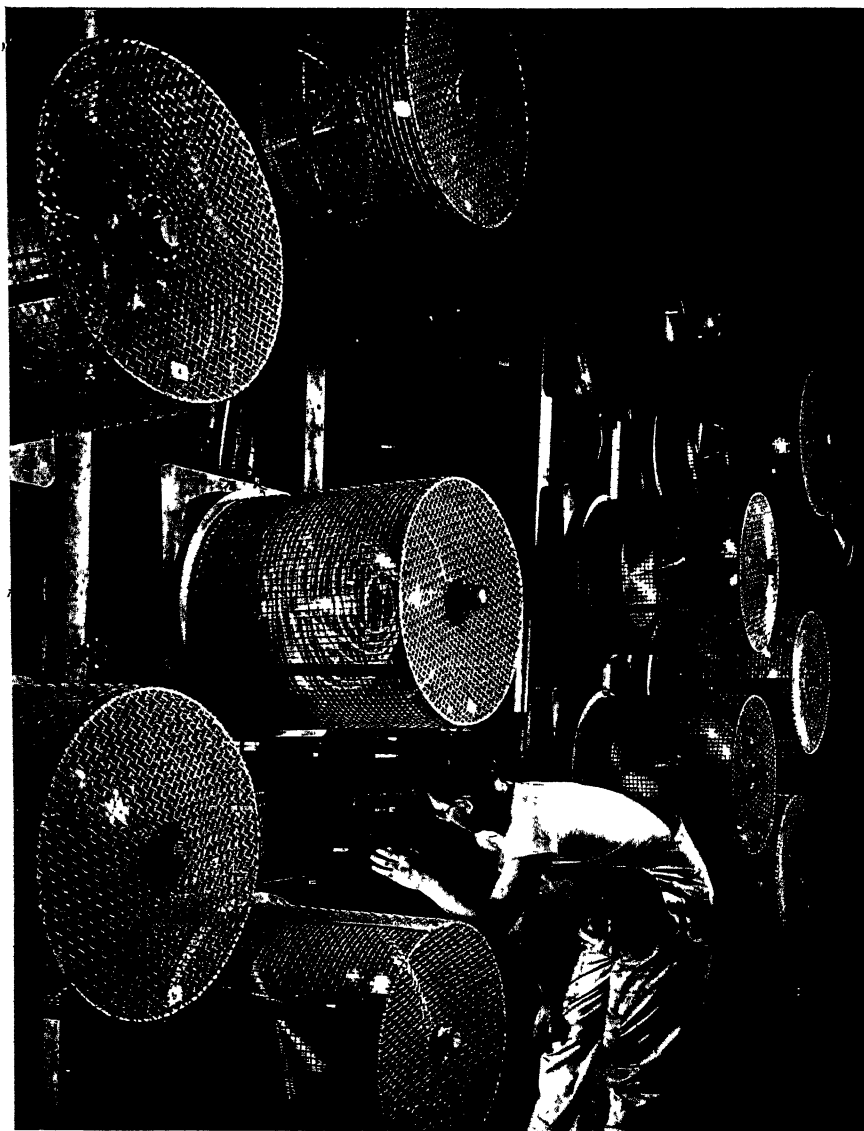


FIG. 15. Small industrial multiplate centrifugal still, with covers and condensers removed

the square root of the temperature and the molecular weights. But superimposed on this is another motion which is the tangential speed with which the molecules are flung from the rotating rim, and this will be the same for all the molecules. Now, in general, the heavy molecules will proceed further along

the tangential path during their passage under random motion than the small molecules will. But at the same time heavy molecules will be generated farther outward on the rim because they will evaporate later. So there will be a tendency for the more volatile materials to collect in an inner zone and the less volatile in an outer zone and this will give an improved separation. There is

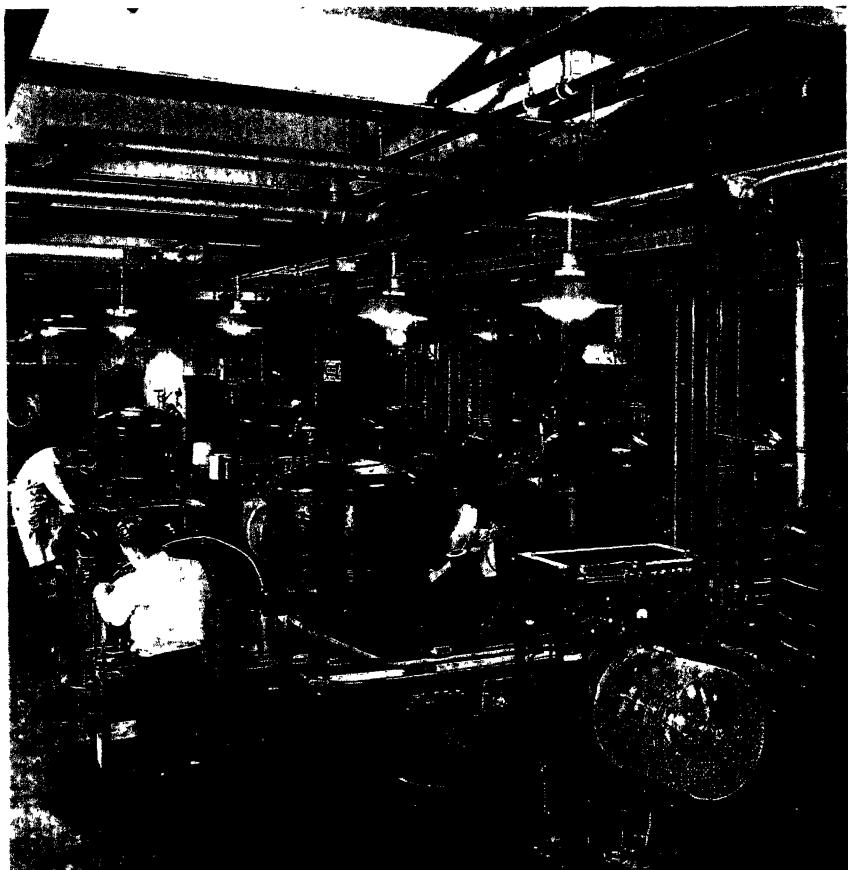


FIG. 16. Installation of larger industrial centrifugal stills; used chiefly for the separation of vitamin A ester concentrates from fish liver oils.

scope for considerable ingenuity in the design of stills in which the rotor is considered as the generator in the molecular-ray apparatus.

V. A COMPARISON OF FILM THICKNESS

The decrease in thickness of distilland, and thus time of exposure, which has come about during the transition from the pot still to the centrifugal is given in table 1. If we assume that a molecule of fat is approximately 5–25 Ångström units long and that a pool of distilland in a pot still is 5 cm. thick, then the dis-

TABLE 1
Transition from pot still to centrifugal still

APPROXIMATE DATE	STILL	APPROXIMATE* DISTILLAND THICKNESS	APPROXIMATE† MOLECULAR THICKNESS	APPROXIMATE TIME OF EXPOSURE
1922	Laboratory pot still (52, 82)	1-5 cm.	5×10^7	1-5 hr.
1928	Laboratory tray still (79)	0.1-1 cm.	5×10^6	5-60 min.
1935	Industrial falling-film still	1-3 mm.	5×10^5	2-10 min.
1930	Laboratory falling-film still	0.1-0.3 mm.	5×10^4	10-50 sec.
1940	Industrial centrifugal still	0.03-0.06 mm.	10,000	0.1-1 sec.
1936	Laboratory centrifugal still	0.01-0.02 mm.	3,000	0.04-0.08 sec.
1942	High-speed centrifugal rim still	0.001-0.005 mm.	400	0.001-0.005 sec.

* Assuming similar throughput for same unit area of all stills.

† Assuming that the molecule of glyceride fat has an effective diameter of 15 Å.

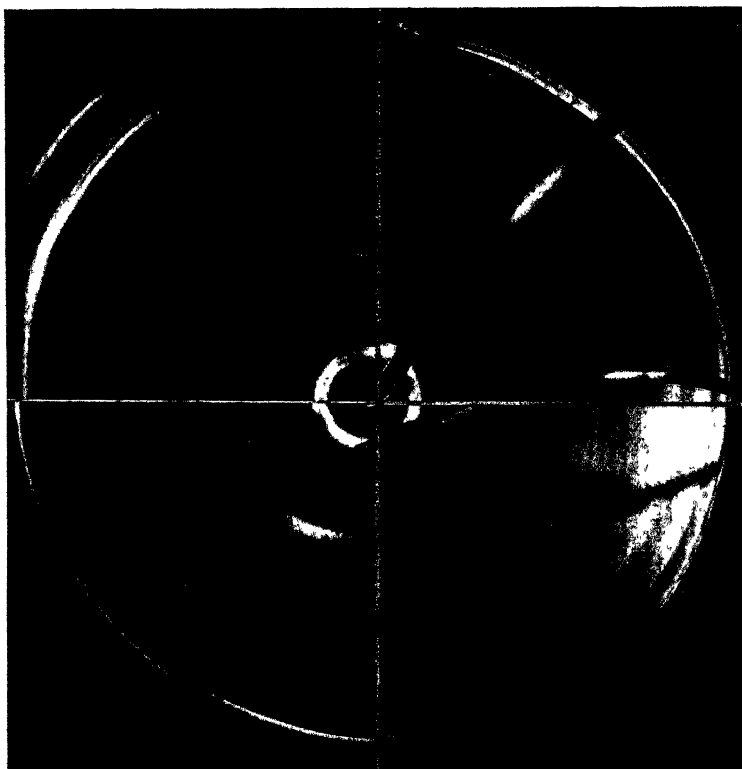


FIG. 17. Quarter sections of four photographs of centrifugal plate with small stream of dyed oil superimposed eccentrically on the main stream: upper left, 250 R.P.M.; upper right, 500 R.P.M.; lower right, 1500 R.P.M.; lower left, 3500 R.P.M.

tilland is some 2-10 million molecules deep. In the present stills the distilland averages 3000 molecules thick, but in the ultracentrifugal still of the future it need not be more than 25-500 molecules thick.

The disposition of the film on falling-film and centrifugal surfaces has been investigated by the use of heavily dyed oils. When an oil, colored so deeply blue that it appears black in a glass tube of 5-mm. bore, is allowed to flow over the surface of a smooth nickel column in a falling-film still, an even deep blue film results. However, after the application of vacuum and heat, the film collects into rivulets showing considerable variations in density. When the dyed oil is passed over the centrifugal surface, the distribution appears uniform



FIG. 17a. Two half-photographs of centrifugal plate with colorless oil flowing across surface and individual drops of dyed oil projected at $\frac{1}{4}$ -sec. intervals; left-hand section, 1500 R.P.M.; right-hand section, 3500 R.P.M.

under conditions so far investigated. The dyed oil has not been photographed above 150°C . because we do not yet have a sufficiently involatile dye of high solubility. The rate of progress down a vertical column when no distillation is occurring is uniform after the oil has attained the temperature of the column, which it does in the first few centimeters of travel, and thereafter the thickness remains constant also. The thickness over the centrifugal surface is not constant, because the oil is being spun thinner and thinner over an ever-widening circle. The progression has been investigated by measuring the reflection density of the oil film at the peak of the dye absorption curve. As an alternative method we have photographed an intermittent stream of colored and colorless

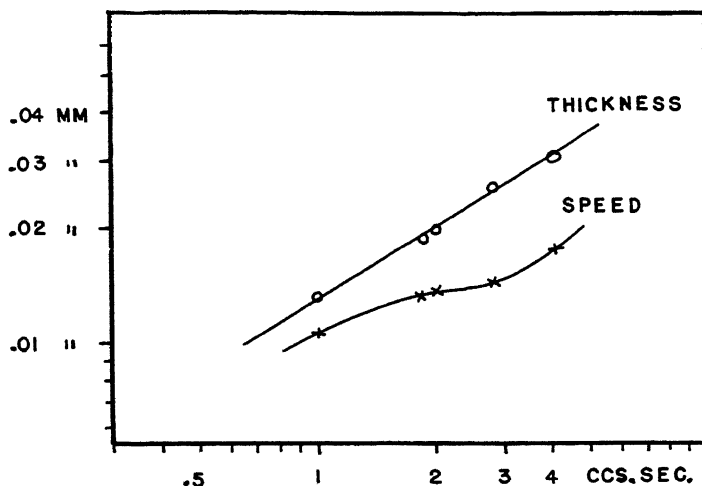


FIG. 18. Chart showing variation of thickness and rate of travel of oil across centrifugal plate with varying speeds of rotation. Measurements taken at 10 cm. from center.

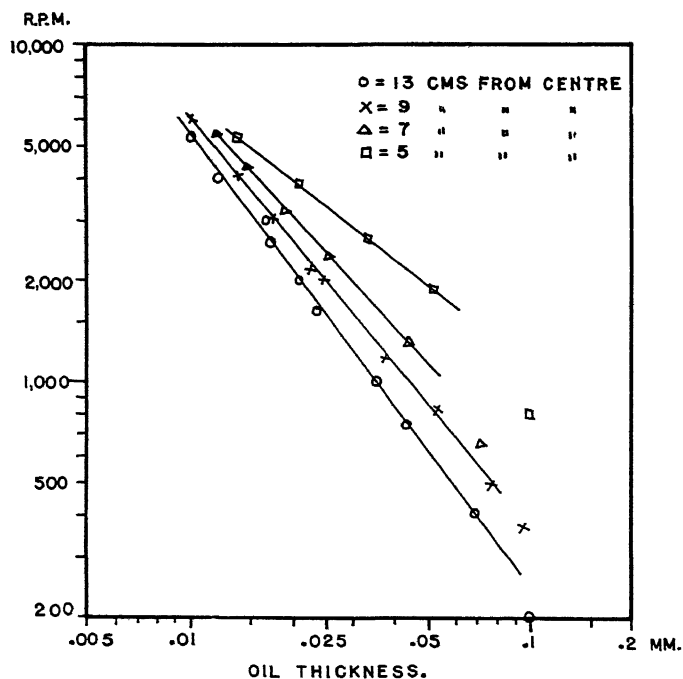


FIG. 19. Chart showing variations of thickness of oil with distance from center and rate of rotation of centrifugal evaporator.

oil fed to the center, in which case the color proceeds outwards in concentric rings. The flow of oil being stratified, an average speed must be interpreted as a faster travel over the outside surface and a slower travel, approaching zero,

against the plate. Since, however, diffusion is rapid compared with the rate of travel in such a thin film and turbulence may be present, we may still speak of the average progress of the film in the belief that this has a true physical meaning. We see from the photographs in figure 17 that the progression is nearly uniform from center to edge, with the thickness diminishing correspondingly with the distance from the center. The ratio of thickness of the oil film and its speed of progression at 100°C. and 3000 R.P.M. 10 cm. from the center of the plate to rate of feed is shown graphically in figure 18. The variation of thickness of film with distance from the center and speed of rotation is shown in figure 19. The logarithm of the speed of rotation is proportional to the inverse log of film thickness over a wide range.

$$\log \text{ R.P.M.} = R \left[\frac{1}{\log \theta} \right] \quad (4)$$

VI. THE QUIET DISTILLAND LAYER

Liquids have been classified as those which evaporate quietly in the molecular still and those which splash and bubble. The latter tendency, mentioned repeatedly (12) in the early literature (14, 15), has been referred to again recently (13). Our own observations suggest that, properly treated, few liquids need show this distressing behavior, which causes contamination of the distillate with distilland, often ruining a distillation. Among the causes of splashing may be listed: (1) imperfect degassing of the distilland (60); (2) fissures and cracks in the hot column or particles of dirt on the surface, all of which afford crevices for decomposition and evolution of gas and are points for detachment of distilland; (3) too rapid evaporation. A liquid evaporating into a vacuum at saturation pressure 5×10^{-3} , which is in the useful operating range, will develop a stream of vapor capable of lifting against gravity from a horizontal surface a layer of oil 0.075 mm. thick, assuming that the distilland is fifteen times lighter than mercury. The smallest pressure would detach the layer from a vertical falling-film or rotating plate were it not for surface tension and the whole range of properties comprised in "wetting." In addition to the evaporation pressure at the outside of the liquid film, there is the potential pressure on the inside against the hot column, which may be considerably higher during rapid distillation. The liquid separates from the column at a fissure or a particle of dirt and when once separated gathers into drops. The drops expose less surface and thus tend to return to the distilland, but they may be prevented from combining by the gas adsorbed to or emanating from the approaching surface. Droplets returning to the hot wet surface at glancing angles may be reflected many times before cohering. Detachment is minimized and reattachment facilitated when there is an external force pressing the liquid against the evaporator. The falling-film column should be a pyramid, broader at the base than at the top, and the centrifugal still should be conical, paraboloid, or cylindrical with the liquid flowing on the inner surface. If the approximate film thickness and speed of rotation are known, the minimum slope contour of the rotor can be calculated

and distillations can be done at high saturation pressures not previously contemplated (62).

VII. RELATIVE STILL PERFORMANCE

There are mechanical, thermal, and other limitations to the rate at which oil can be put across a surface still. Excessive throughput impairs the vacuum, overtaxes the heat transfer, and spoils the degree of separation. The ability to separate constituents from a mixed distilland is a fundamental property of a still. Classically, this would be investigated by measurement on distillates from a binary mixture. A method more in accordance with the technique from this laboratory is the examination of the shape of the elimination curve from a cyclic distillation. The generation of the curve is given in Part II, to which the reader is now referred.

The sharpness of separation can be expressed by the elimination coefficient ϵ , given by the ratio of the height to the area under the curve obtained when yield,

TABLE 2
Elimination coefficient with different stills

STILL	APPROXIMATE ELIMINATION COEFFICIENT	APPROXIMATE FRACTION SURVIVING		
		β -Carotene	Vitamin A palmitate*	Vitamin D palmitate*
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Wide-necked alembic flask (figure 2).....	0.7†	0	10-20	0
Pot still.....	0.6	20	80	0- 5
Falling-film still (laboratory).....	0.9	50	98	10-30
Centrifugal still (laboratory).....	1.1	90-95	100	75-98

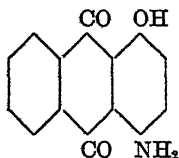
* Natural esters of C_{16} , C_{18} , C_{20} , and C_{22} acids, C_{18} predominating.

† With ebullition or mechanical stirring.

in successive fractions of a chosen constituent of a mixture, is plotted against temperature, in rigidly defined units.⁵ The optimum and limiting value for a very slow distillation of a polar dye from a polar oil⁶ is approximately $\epsilon = 1$. As the throughput is increased the coefficient decreases to 0.7-0.6, at which point vacuum impairment, splashing, and other factors begin to interfere. Distillations of fat-soluble vitamins from non-polar oils have been done with $\epsilon = 1.1$ -1.2. Conditions which yield an elimination coefficient of 0.6 in the pot still and 0.7 in the falling-film still will often give $\epsilon = 1.1$ in the centrifugal. The survival of labile substances goes hand-in-hand with high ϵ values, as shown in table 2.

⁵ See page 87 *et seq.*

⁶ For instance, Celanthrene Red from glyceride constant-yield oil.



A. Fractionation

Separations better than are obtainable with a power of one theoretical plate can be secured by multiple distillation. When this enhanced separation is performed in one coordinated act, the term *fractionation* may be used. Fractionation by *feed-back* during passage of the distilland over a series of molecular stills has been covered by Fawcett (40) and Fraser (42). There are, however, so many other mechanical ways of inducing high-vacuum fractionation that a description of some of these must be left to a future paper.

B. Rate of distillation

Factors 2 and 3 (page 59),—namely, *distillation from the surface* and *travel across the vapor gap*,—will now be examined.

Two classes of molecules contribute to the pressure in a surface still—the residual gas and the distillate vapor. The pressures are referred to as *residual pressure* and *saturation pressure*. The mass, W , in grams evaporating per second per square centimeter of substance of molecular weight M , at absolute temperature T and saturation pressure P (in millimeters of mercury) or p (in microns of mercury), is

$$W = 5.83 \times 10^{-2} \cdot P \cdot \sqrt{\frac{M}{T}}$$

and

$$p = \frac{17,200 \cdot W}{\text{Area cm.}^2} \cdot \sqrt{\frac{T}{M}}$$

It so happens that $\sqrt{\frac{M}{T}}$ approximates unity. Thus, T for distillation at 1 micron varies from about 350°K. for palmitic and stearic acids to about 550°K. for tristearin and from 570 to 580°K. for castor oil and the harder waxes. The molecular weight varies from 284 for stearic acid to 850–950 for glyceride fat.

$\frac{M}{T}$ varies from 0.9 to 1.3, the sterols having a factor of nearly unity. It is convenient to remember that at a saturation pressure of 1 micron (0.001 mm.), 1 square meter will evaporate approximately 0.5 g. of stearic or palmitic acid, 0.55 g. of sterols, and 0.75 g. of triglyceride fats or oil each second.

The rate of collection of distillate approaches the rate of evaporation only at zero residual pressure and a short unobstructed path. With wide gaps and substantial residual pressures few molecules will reach the condenser without collision and many will be returned to the distilland. Burch (British patent 303, 078 (see page 00)) predicts the fate of a mercury molecule in residual gas at 1 micron with distillation gaps of various widths, as shown in table 3, and hazards the guess that a distillation which is vigorous with a 2-cm. gap will be virtually stopped with ten times the gap. Experience teaches that distillation is less hindered by residual gas than is suggested by simple kinetic theory. The resi-

dual molecules are not an independent maze through which the vapor must wander, but a movable barrier readily disturbed by distillation. Transfer by diffusion is aided by convection currents impressed by the evaporating molecules, which induce a *draught* towards the condenser even when they are in the minority. When they are in the majority they drive all the residual gas towards the condenser and thence sideways to the edge of the gap. Under practical operating conditions (1-10 microns) there is a gradual increase in the rate of distillation as saturation overtakes residual pressure. The variation of rate of

TABLE 3
Fate of mercury molecules in residual gas at 1 micron with distillation gaps of various widths

DISTANCE BETWEEN EVAPORATOR AND CONDENSER	MULTIPLES OF MEAN FREE PATH	PROBABLE FRACTION OF MOLECULES MAKING JOURNEY WITHOUT COLLISION
<i>cm.</i>		<i>per cent</i>
1	0.25	77.5
2	0.5	60.1
20	5.0	0.005

TABLE 4
Variation of rate of distillation with pressure

PRESSURE OF RESIDUAL GAS (AIR)	RATE OF DISTILLATION PER SQUARE METER OF DISTILLAND SURFACE OF FALLING-FILM STILL (2-CM. GAP)		
	Saturation pressure of distilland (2-ethylhexyl phthalate or Octoil)		
	1 μ	3 μ	10 μ
μ	<i>grams per second</i>	<i>grams per second</i>	<i>grams per second</i>
0.3	0.6	1.85	6.4
4.0	0.46	1.59	5.7
7.0	0.38	1.37	5.2
10.0	0.32	1.18	4.6
15.0	0.25	0.95	3.8
25.0	0.21	0.70	2.1
50.0	0.12	0.40	1.67

distillation at three saturation pressures and varying residual pressure in a falling-film still is given in table 4.

c. Projective vs. equilibrant distillation

Under molecular conditions of direct transfer to the condenser, molecules do not reënter the distilland and there is no equilibrium between liquid and vapor. The quantity distilling is proportional to $P/\sqrt{M}$ and the relative quantities of two or more constituents are

$$\frac{p_1}{\sqrt{M_1}}, \frac{p_2}{\sqrt{M_2}}, \dots, \frac{p_n}{\sqrt{M_n}}$$

In ordinary distillation, where the rate of escape of vapor is less than the rate of generation, molecules reënter the surface and produce approximate equilibrium between liquid and vapor phases. The quantities of constituents distilling are then proportional to their partial pressures, $p, p_2 \dots p_n$.

Molecular distillation will thus effect separation between substances of equal vapor pressure, providing the molecular weights are different. On the other hand, substances having

$$\frac{p_1}{\sqrt{M_1}} = \frac{p_2}{\sqrt{M_2}}$$

which are inseparable molecularly, can be separated if conditions of partial equilibrium can be tolerated at the necessarily higher temperature. A mixture can sometimes be resolved by distilling first under projective and then under equilibrant conditions.⁷

The unique kind of separation afforded by the molecular still was recognized by Brönsted and Hevesy (10) and has been featured by other writers (12, 13). It was early noted that the $P/\sqrt{M}$ type of separations persisted at saturation pressures far exceeding the truly "molecular." The controlling factor would seem to be less the density of the advancing vapor, where collisions are of "the same sort," than the opposition offered to the vapor by hot mechanical obstructions or by residual gases which oppose collisions of "a different sort." The degree of equilibrium, therefore, is determined by the ratio of the rate of *reception* to the rate of generation of the vapor, and projective separation could be secured at extremely high saturation pressures if these could be generated without disruption of the distilland and if the vapor could escape and be absorbed without hindrance. Unobstructed-path distillation at high saturation pressure is feasible and commercially attractive in centrifugal apparatus (62). Conversely, more or less equilibrium distillation can be established even at the lowest saturation pressure by controlling the residual pressure or the temperature of the condenser, or both. By maintaining a relatively warm condenser, equilibrium distillations can be performed in the molecular still at the lowest temperatures compatible with the degree of obstruction.

D. Partial condensation

Partial condensation is a well-known aid to separation and is useful because it adds to the selective power exerted at the evaporating surface a second selection by the condenser. It can not be employed in the simple molecular still without banishing the very conditions under which the still was built to operate. In diagram A, figure 20, the solid arrows represent heavy molecules readily absorbed by the hot condenser, and the dotted arrows represent light molecules chiefly rejected. Neglecting the possible escape sideways to the pumps, the light molecules must return to the evaporator, enriching the distilland until so many can be thrust towards the condenser that the small proportion retained

⁷ The difference between the two kinds of distillation is small and its importance is apt to be exaggerated.

balances the input of the constituent to the still. Note that the steady composition of the distillate is the *same*⁸ as with a cooled condenser, but distillation is hindered both by the molecules uselessly cruising about the gap and by the number of false strikes before condensation—it is no longer a molecular distillation.

To utilize the advantages of a warm condenser the cruising light fraction must be allowed to escape, while the heavier molecules are condensed. This is done

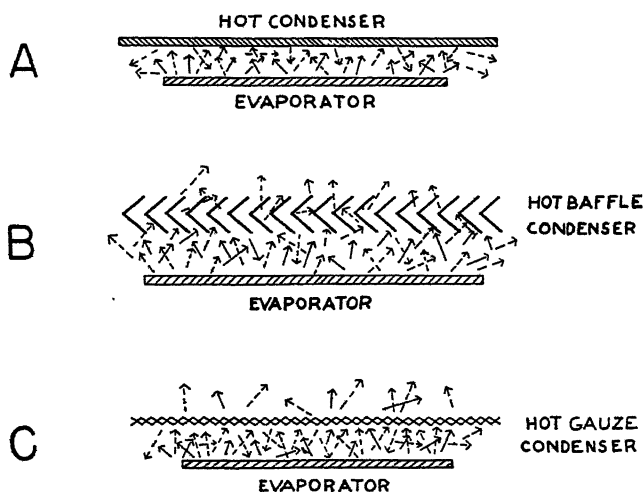


FIG. 20. Fractionation by partial condensation: A, with hot barrier; B, with barrier condenser; C, with semi-permeable film barrier.

TABLE 5
Typical losses during a molecular distillation of triglyceride fat

	FRACTION OF HEAT CONSUMED
	<i>per cent</i>
Conduction through body of still.....	6.2
Heating oil to distillation temperature.....	35.3
Radiation losses.....	54.1
Latent heat of distillation.....	2.0
Total accounted for.....	97.6

by a perforated construction (71) which may be open, as in B, figure 20, or closed by a film of condensate, as in the wire-mesh construction or C, figure 20. Here again distillation is partially unobstructed and partially equilibrant.

The idea of the self-pumping still has intrigued inventors from the first. In a multi-evaporator still, Burch (14) allowed the more volatile constituents of a mixture to provide the vacuum for the less volatile. Van Dijk (96) went further and grouped parts of distilling and evaporating plates so that the ma-

⁸ Except for the small change in *kind* produced by the approach to equilibrium condition.

jority of the inevitable collisions between evaporating molecules and residual gas is towards the exit to the pump.

These devices are a fundamental advance in design. When misapplied they involve a contradiction in terms, since they attempt to utilize collision to produce a vacuum in which there are no collisions,—the vacuum is to be lifted by its own bootstraps, so to speak. Recent self-pumping stills utilize the heat of condensation (65) and other spare energy to operate molecular or condensation pumps within the still. The value of a pump situated *within* the still appears to have been recognized first by Vigers (98).

E. Thermal balance in the molecular still

The molecular still has a naturally low thermal efficiency because the hot evaporator faces and is coextensive with the cooled condenser. Fawcett (38)

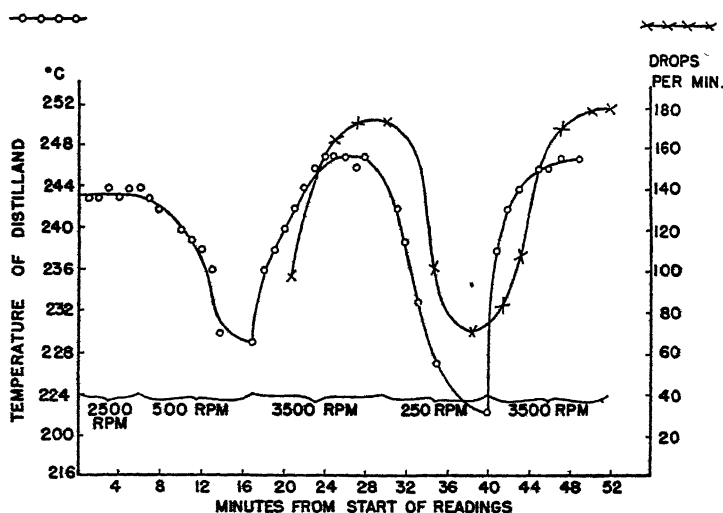


FIG. 21. Chart showing increase in temperature of distilland and rate of evaporation with increase in rate of rotation of evaporator.

has listed typical losses during a molecular distillation of triglyceride fat as shown in table 5.

Only about 2 per cent of the heat is actually used for the distillation, the rest being spent for contributory purposes. Of the fraction heating the oil some can be recovered by countercurrent flow, but this leaves radiation stealing an even greater share. Polished baffles (109) between condenser and evaporator have been proposed to reflect part of the heat, but these obstruct the path if hot and are inoperative if cold. The reason for the failure of a cold reflector is that the prime radiator of heat in the molecular still is not necessarily the hot distilling column, but is the oil on its surface. Glyceride oils are substantially opaque to radiation from bodies at 250–350°C. Even though the evaporator and condenser can be made of highly polished heat-reflecting metal, the hot oil on the

evaporator and the cold condensate on the condenser are the prime bodies emitting and receiving heat. The centrifugal still offers some hope for heat economy, because the distilland can be whirled so thin that it ceases to emit radiation. In a like manner the distillate can be whirled thin on an opposing condensing member, and if the base metals are maintained bright, little heat is emitted and that which is emitted is largely reflected whence it came. This effect has been measured in a number of ways, of which the most convincing is the increase in the rate of distillation with increase in speed of rotation of the distilling surface. This is shown in figure 21, where the history of a distillation done at constant heat input and varying rotational speeds is plotted graphically. A small part of the increase at high R.P.M. is due to increased convection in the vapor phase. We have not yet had the opportunity of repeating the measurements with a highly reflecting rotating condenser. It is evident that the use of *one* reflecting surface, either distilling or condensing, affords the greatest step in energy conservation, the second reflector effecting a minor saving.

VIII. CONTRIBUTORY TECHNIQUE

A. *Vacuum pumps*

The volumetric capacity of vacuum pumps required by a molecular still measured at the operating pressure (0.1–5 microns) is relatively enormous, many thousands of liters of gas having to be withdrawn for each liter of oil entering the distilling zone. There is no inherent reason why large mechanical pumps should not be devised to perform this evacuation efficiently but, as a matter of history, the larger molecular stills have been evacuated by Langmuir's condensation pumps. Early distillations were done with the mercury pump, but as apparatus increased in size—and particularly after Burch's introduction of the Apiezon oils—it was found more satisfactory to use organic liquids in the pump. One reason is the poisonous character of mercury; another is the tendency of the condensed mercury to become inextricably emulsified with the oily distillate withdrawn from the still by the pump.

The principle of the condensation pump is well known but will be indicated here for the sake of completeness. Vapor issues from an orifice attached to a shallow boiler. The orifice may face upwards as in A, figure 22, but is generally turned downwards towards the boiler as in B. In any case the orifice or "jet" is surrounded by a water-cooled jacket, one end of which serves as the suction intake, while the other serves as the discharge lead. The pump is a secondary or "booster" device which requires a primary pump to bring the effluent up to atmospheric pressure. It is the function of the primary pump, which may be mechanical⁹ or a multi-stage steam ejector (57), to create a vacuum of 0.3–0.05 mm. into which the condensation pump is able to discharge. The vapor pump has an enormously greater volumetric capacity than the fore pump, but the mass of gas (PV), is identical for each of the two pumps in tandem. Indeed, the *re-*

⁹ Such as the well-known Cenco Hyvac or Megavac and the larger Kinney or Stokes pumps. Many other excellent makes are available.

serve mass capacity, or liter-micron-capacity as it has been called by the British group, must always be larger in the prime mover, which will otherwise be unable to handle the gas gathered and compressed by the condensation pump. An example will make this clear:

A condensation pump is required to handle 100 liters per second at 10^{-4}

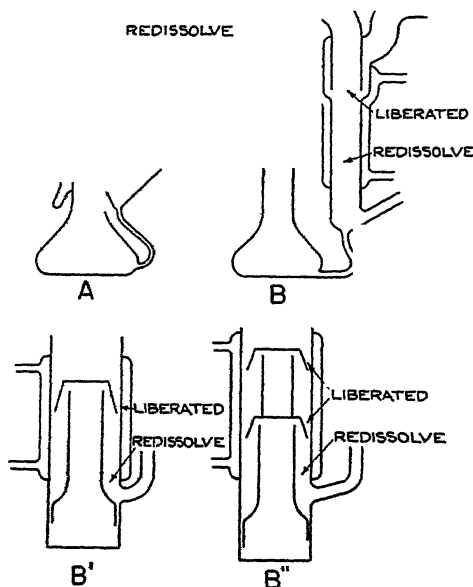


FIG. 22. Diagram illustrating contamination of condensation pumps

mm., which it will discharge into a mechanical fore pump at 0.1 mm. pressure. The volume of gas handled at higher pressure will be

$$\frac{100 \times 10^{-4}}{10^{-1}} \text{ liters} = 100 \text{ cc. per second}$$

This can be accommodated by the smallest laboratory mechanical oil pump. However, it will be necessary for the mechanical pump to handle larger quantities if the system is to begin operating within a reasonable time. Assuming that the condensation pump reaches its full speed at 5×10^{-3} mm., the capacity of the fore pump should be at least

$$\frac{100 \times 0.005}{0.1} = 5 \text{ liters per second}$$

Thus, in the interests of quick starting the vacuum prime mover may well have a capacity twenty to one hundred times that needed for ultimate maintenance.

In addition to a capacity for handling permanent gases, the pump attached to a molecular still should dispose of semivolatile materials. Organic fluids

differ from mercury in that permanent gases are slightly soluble and vapors are extremely soluble in them and may form constant-boiling mixtures. The pump fluid, therefore, continually becomes contaminated (51) by the very materials which it should reject, and the speed of the pump diminishes correspondingly (see regions marked "liberated" and "redissolves" in figure 22). To overcome this, self-purifying pumps (17, 58, 86) have been devised which use a plurality of boilers in series, each boiler rejecting partially the impurities left over from the preceding boiler. In practice, three to four boilers (53) have proved sufficient for the industrial falling-film stills. With the continued growth of molecular distillation equipment it would appear that larger fractionating pumps must be devised or perhaps a complete purification plant will be added to pumps of more conventional design.

B. Pressure measurements

Two pressures only would seem to be of importance in the short-path still. One is the saturation pressure of the distillate vapor with which we have dealt previously; the other the pressure of residual gas. The pressure of residual gas is a measure of the equilibrium existing between leakage and decomposition on one hand and the sum of the "admittances" of the pumps and ducts on the other. The necessity for large ducts is indicated by the chart reproduced, as figure 23, by the courtesy of the General Electric Company Laboratory. For a fuller discussion of the problem of gas flow the reader is referred to the original work of Knudsen (76, 78) and the standard textbooks (75, 84), especially those of Dushman (27), Dunoyer (26), and Strong (94).

It is unnecessary to know the residual pressure unless this is harmfully high. It is the object of the distillation technologists to reduce the pressure to such a level that it is negligible. To do this it is imperative to use gauges which do not minimize the pressure in the still. Of the many gauges which are available, including the Pirani hot-wire, the ionization, the Knudsen, the oil manometer, and the McLeod, the latter is least suited for the purpose owing to its failure to register condensable vapors. The Pirani, in spite of criticism (37), remains our choice. The gauge requires constant checking for shift of zero point. It is advisable to have a number of instruments available so that those not in use can be returned to the calibration rack, where they are compared with a McLeod gauge which is operating properly upon residual air instead of vapors. The hot-wire gauge used in this manner becomes eminently satisfactory and has the merit that when it eventually fails it exaggerates the pressure in the still. The pressures which should obtain in the still are preferably less than 10^{-3} in laboratory stills and 5×10^{-4} in industrial stills.

Another gauge which is valuable is the tilted U-tube manometer filled with amyl phthalate (density = 1 at 25°C.). This is used as a double deflection instrument by placing one limb in connection with the vacuum to be measured, while the other is connected with a good reference vacuum. The connections to the two limbs are then reversed and the difference between the two readings—halved—yields the true pressure, compensated for the usual errors of the method.

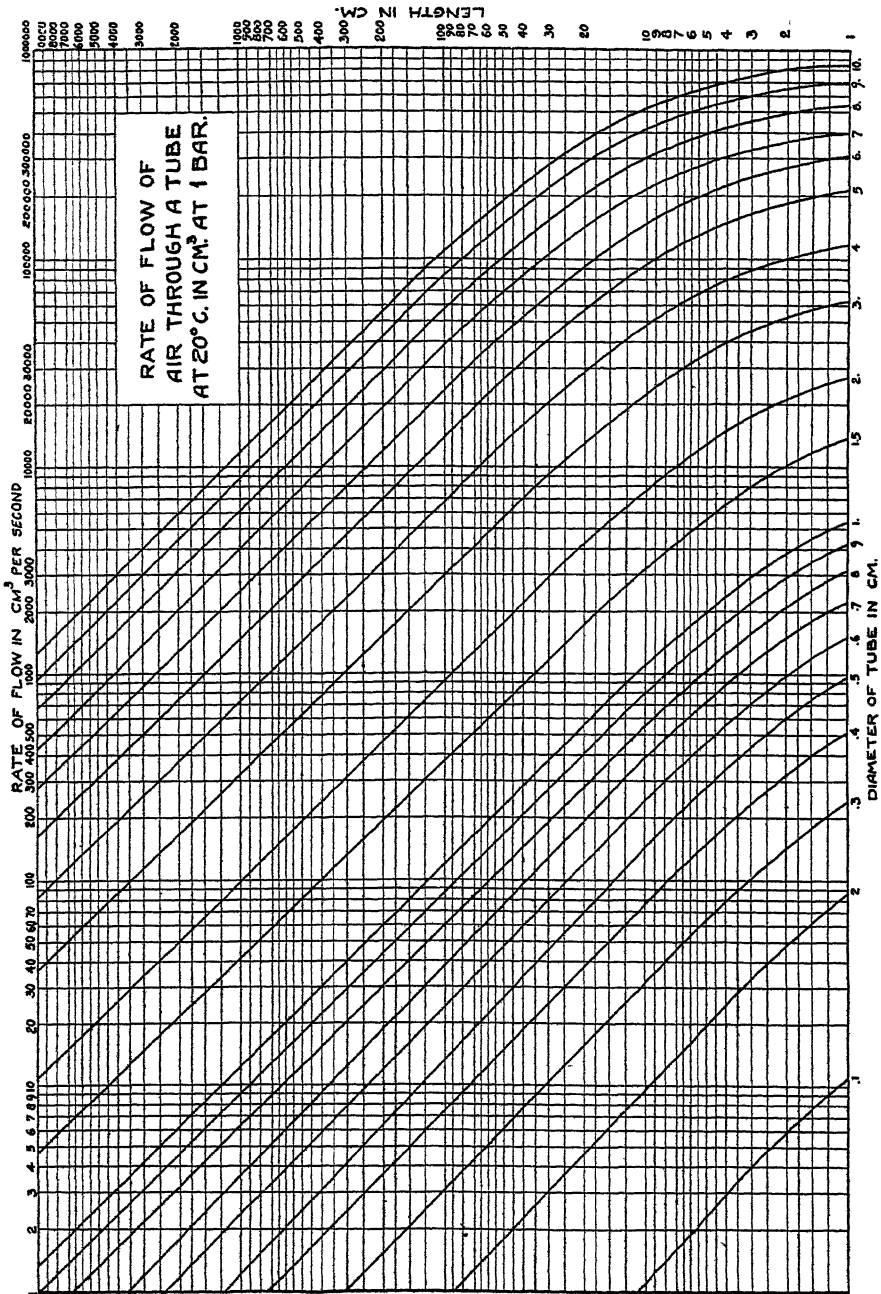


Fig. 23. Diagram showing variation in rate of flow of air through a tube at 20°C and 1 bar. (Courtesy of General Electric Company, Schenectady, N. Y.)

Absolute pressures down to 10 microns can be obtained which are useful in calibrating the Pirani instrument.

IX. FUTURE AND SCOPE OF SHORT-PATH DISTILLATION

Hitherto molecular distillation has succeeded industrially with valuable materials that can bear high process costs and with materials having constituents of such different volatilities that the poor separating power of the stills has been unimportant. Potent fish liver oils have been admirable candidates for distillation. Indeed, had it not been for the timely rise of the vitamin industry, short-path distillation might have languished in the limbo of forgotten inventions. The demand for vitamin A provided a use for the early stills, as well as funds and incentive for the development of larger more economical models.

Scientifically, the graduation of the molecular still from a capacity of a few drops to the present aggregate of about five million pounds annually represents the major developmental span. Industrially, the main period of development lies ahead. The natural oils and waxes which form one of the world's last great reservoirs of unprocessed organic raw material should be the source of a billion or more pounds of molecular distilland. From the heavy chemical industries plasticizers, plastics, resins, polymers, highly oxygenated glycols and sugar derivatives, rubber intermediates, synthetic lubricants, and petroleum residues head a long list of chemicals amenable to high-vacuum purification. No matter how complex the product, the last stage of synthesis generally involves the type reaction $A + B \rightarrow AB$, where A and B are intermediates of low or medium molecular weight and AB is of medium or high molecular weight. In either case A and B differ vastly in volatility from AB, so that excellent separations can be secured in a single passage through the short-path still. In some cases both reactant and product can be distilled in succeeding evaporators; in others the product can be stripped of reactants while itself remaining undistillable. In either event the excess of the original components can be returned to the reaction kettle to augment succeeding batches. At present they are wasted as a contaminant of the product, the properties of which are often impaired.

The future of the molecular still has sometimes been viewed with pessimism (13) because of high thermal requirements and ineffectiveness in separating the largest class of raw materials—the natural mixed triglycerides. However, there are ways of economizing in heat, as we have shown, and heat becomes cheaper each year. Indeed, the preëminent advantage of the molecular still in the oil refinery is that heat is the only contributory raw material needed. The separation of the mixed glycerides will probably wait upon the development of the *fractionating* wide-path still. In the meantime the stripping of vitamins, sterols, and volatiles from the natural oils may well occupy a decade of industrial development.

PART II

I. APPLICATIONS OF MOLECULAR DISTILLATION

The applications of the short-path still are as wide as the literature, which includes more than a hundred papers. Limitations both of space and of the

author's experience account for the emphasis which has been laid on the work of the Distillation Products laboratory in what follows.

Instructions for handling small quantities of oils, sterols (54), dyes, biologicals (55), and volatile solids have been given by McDonald (85) and others. Papers by McDonald, Almquist (2), Dam (22), and Carothers and Julian Hill (19), severally, are worthy of study. Whatever the conditions, the procedure is the

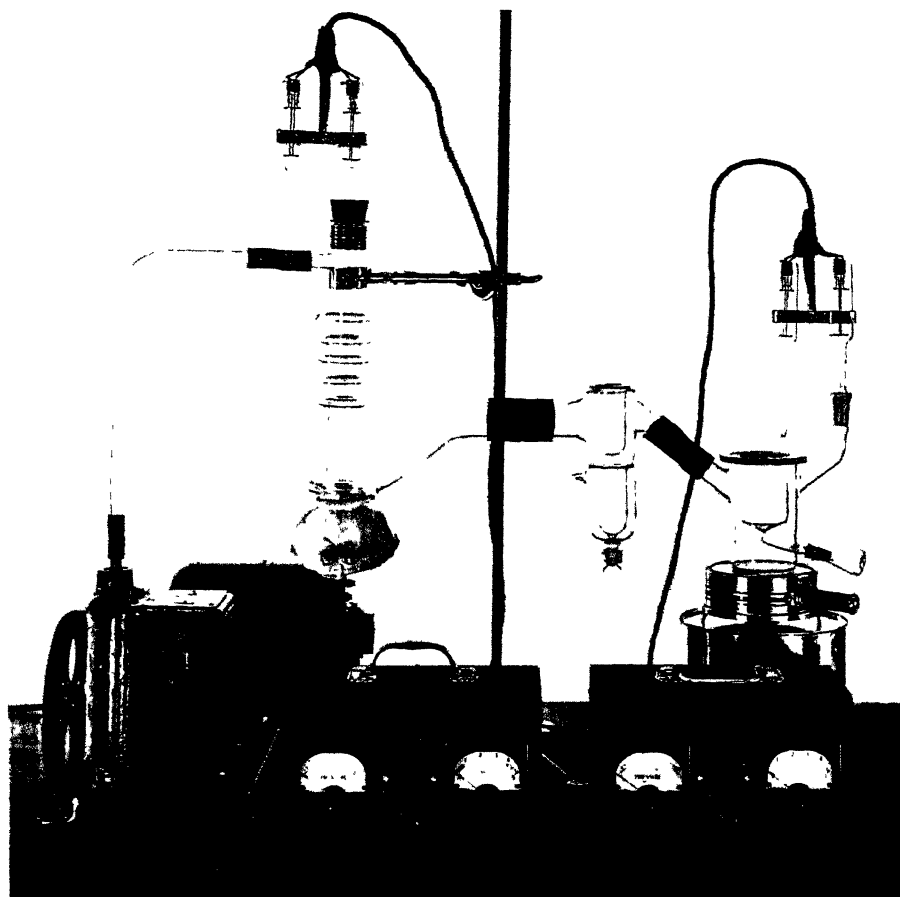


FIG. 24. Simple molecular pot still showing, from right to left, glass pot still with Pirani gauge, low-temperature trap, condensation pump, Pirani gauge for forepressure, mechanical vacuum pump.

same; the substance is placed in the still, a residual pressure of 10 microns or less is established, heat is applied, and a *number of fractions* is collected. A photograph of a simple assembly of apparatus is shown in figure 24. A more ambitious installation of many small stills, treated as permanent laboratory fixtures, has just been described by Riegel, Beiswanger, and Lanzl (92). The photographs reproduced as figure 24a are by the courtesy of these authors. The degree of separation of constituents is poor, generally less than a single theoretical "plate,"

and there is no sharply defined boiling point to guide the operator, who is thus hampered in selecting the best temperature range for collecting fractions. This

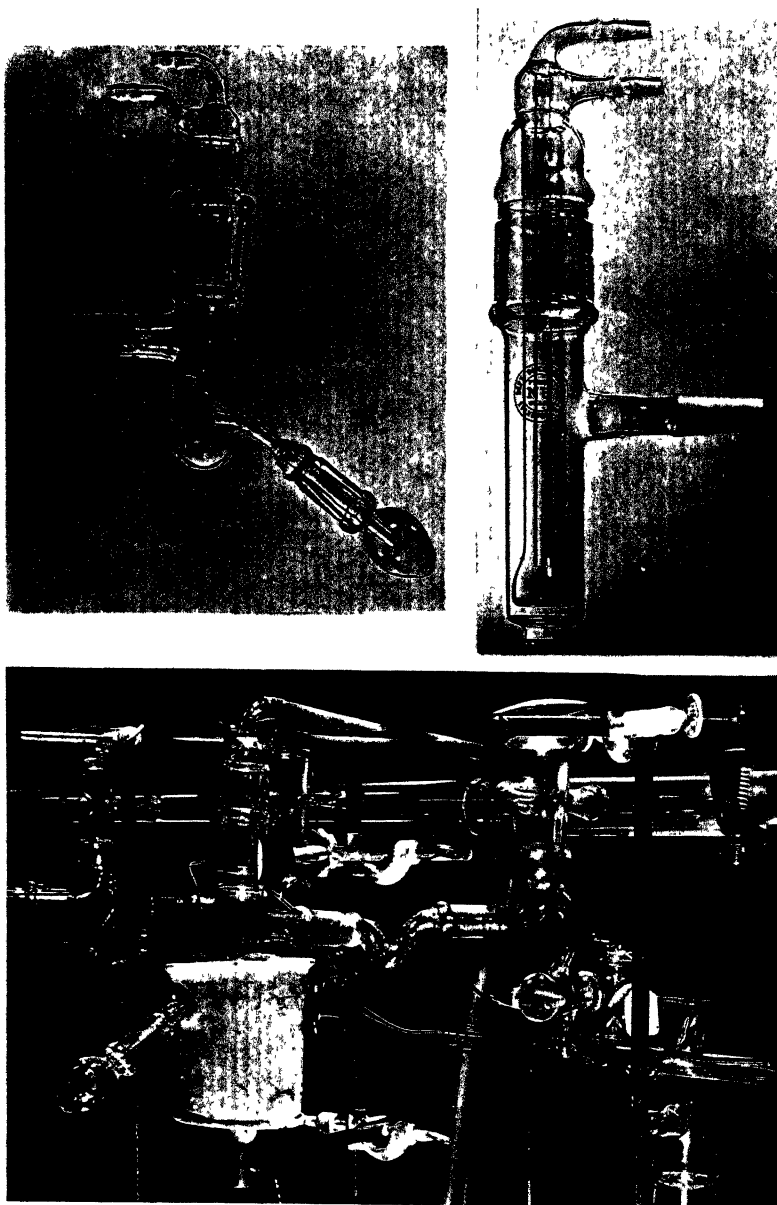


FIG. 24a. Installation of miniature molecular stills after Riegel *et al.* (photographs by courtesy of the authors).

indefiniteness has hindered the wide adoption of molecular distillation in the organic laboratory.

The boiling point of a substance is the temperature at which the pressure of the vapor overcomes that of the surrounding gas. In the molecular still there is no surrounding gas and hence no boiling point, distillation occurring in some degree whenever there is a difference of temperature between evaporator and condenser. Increase in the absolute temperature and in the difference increases the rate of evaporation, but there is no abrupt transition for observation and record. The problem has been to provide a practical substitute for the boiling point.

It was early noted that the change in concentration of a constituent in a sequence of fractions lay on a smooth curve having a well-defined maximum. At low temperatures little of the constituent would collect, but as the temperature was advanced, distillation of the constituent would increase, reaching a maximum only to fall again in later fractions as the distilland became impoverished. It appeared probable that each substance in a mixture would come to maximum yield in fixed relative order which would be unaffected by changes in the absolute rate of distillation. Then, by determining the position of the *elimination maximum*, that is, the fraction richest in any one constituent, it should be possible to predict which of the other fractions contained the greatest yields of accompanying substances. The observed constituent would be referred to as the distillation pilot (52). Volatile dyes were an obvious choice as visible pilots.

Many of the valuable constituents of natural mixtures are present in traces only. Free fatty acids, vitamins, sterols, and hormones are contained in large relative volumes of non-volatile fat. When these are treated in the molecular still the minor constituents collect as a mist on the condenser, from which they can not be removed without interrupting distillation. It was found convenient to add to the distilland a mixture of volatile oils which would distill with the minor constituents and give them sufficient volume to flow from the apparatus without interrupting distillation. The distilland was thus doctored with a pilot dye and a *controlled-yield oil* (6), so that all the significant fractions should have approximately similar bulk and be subject to about the same drainage errors. N. Embree (28) pointed out that if the time for collecting each fraction were kept constant and the temperature raised by uniform increments, the distillation curves would be accurately reproducible and could be employed without pilot dyes. This has proved to be the case and the method of the *elimination curve*, figure 25, has become pivotal for our researches with the molecular still. As a practical matter the pilot technique has been retained, extremely accurate comparisons between the elimination curves of pilot dyes and the accompanying substances being realized. Indeed, relative elimination maxima can be determined in the range 100–200°C. with a precision of $\pm 1^\circ\text{C}$., which compares satisfactorily with ordinary boiling-point measurements.

The elimination technique does not require special apparatus, but it makes heavy demands on the operator who with clock and thermometer must give the distillation undivided attention until the last fraction has been collected.

At present the most convenient type of apparatus for these analytical distillations is a circulating batch still. The falling-film cyclic still has been described many times (52, 54), and a photograph of it (figure 26) is included here merely

for completeness. The distilland is circulated over a small vertical column at a constant rate, and fractions are removed periodically as the temperature is allowed to rise. The still accommodates from 50 to 500 cc. of distilland. A larger model holding 15 liters and equipped with a centrifugal evaporator is shown in figure 27. A later version (figure 28), built and largely designed by F. R. Kraselt, embodies automatic means for advancing the temperature and collecting distillates. The operator places the mixture of distilland, constant-yield oil, and pilot dye in the reservoir, A, arranges fifty matched test tubes in the rotating nest, B, replaces the glass dome, C, and starts the operating clock, D. About 4 hr. later the clock has shut off the pump and heaters, after which the dome may be lifted and the test tubes removed for analysis. A still is under construction

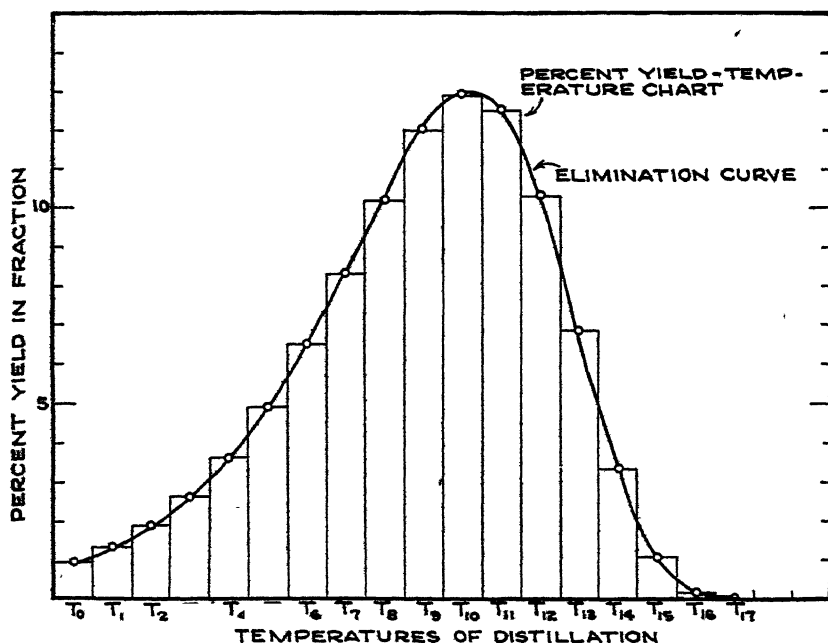


FIG. 25. Theoretical elimination curve, after Embree

which will present a series of individual condensers to the evaporator, thus obviating the need for constant-yield oil. An excellent analytical still of simple construction which can dispense with constant-yield oil has recently been described by Quackenbush and Steenbock (91). Concentric evaporators and condensers are employed, but the latter are on the *inside*, providing a smaller draining surface which, if necessary, can be *wiped* clean. The temperature of the evaporator is accurately controlled by vapor heating.

The elimination technique provides essentially practical information—how substances can be separated from the mixtures in which they occur. Consider the simple case of oleic and stearic acids. From their relative vapor pressures *unmixed* it could be deduced, according to Fawcett (37), that a half-and-half

mixture after a single distillation which removed a quarter of the bulk would give a distillate having the composition 68 per cent oleic acid and 32 per cent stearic acid. But this does not take into account the effect of each acid on the vapor pressure of the other, so that a practical distillation gives the quite different ratio 36 per cent oleic acid and 64 per cent stearic acid, *which could not readily*

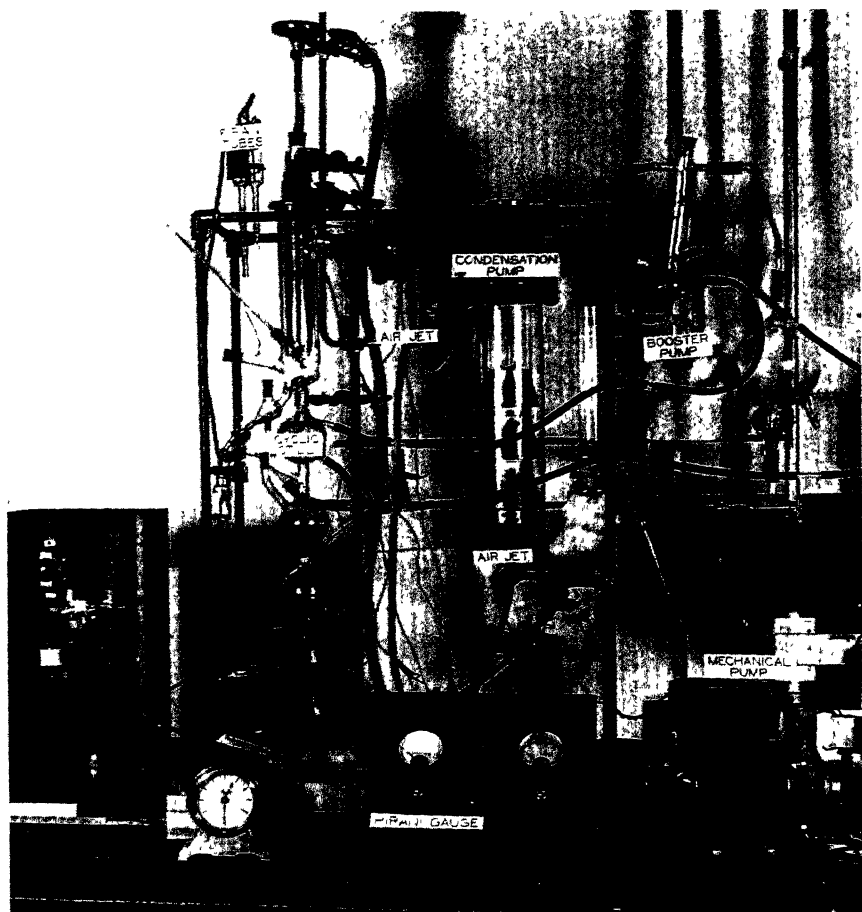


FIG. 26. Cyclic molecular still assembly

have been predicted. It is unlikely that anyone would attempt to separate these pure acids by distillation, crystallization being a better method. But it might well be desirable to distill traces of the two acids separately from a rancid oil. By plotting the elimination curves one can learn at a glance how to conduct a simpler type of distillation to secure the best separation.¹⁰

¹⁰ Our argument, in effect, recommends a practical determination instead of a theoretical approach based on related data. Of course, Fawcett also made the practical measurement, thus furnishing the text for this paragraph. The stress laid on the elimination technique is a plea for a systematized experimental approach which will yield readily recognizable data.

The elimination-curve theory takes into consideration only the mole concentration of the constituents. There are, in addition, their relative affinities for the distilland and for one another,—all those forces vaguely referred to as “asso-

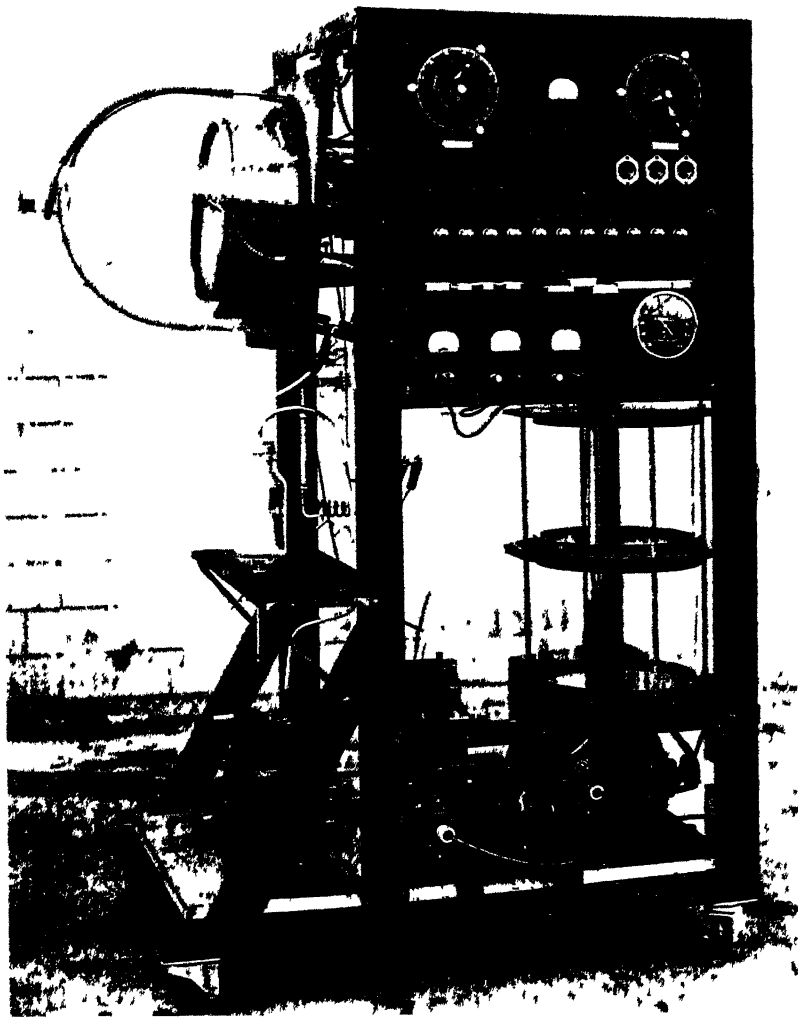


FIG. 27. Cyclic molecular still; large batch type with centrifugal evaporator

ciation,” which cause partial pressures to follow Henry’s law instead of Raoult’s. In the study of natural mixtures of similar origin, for instance, various samples of soybean oil, the association phenomena remain substantially the same from one distillation to another and the elimination curves of the substances under study maintain their accustomed shape and position. But let the solute be transferred to a different substrate, as when a concentrate of soybean non-

saponifiabiles is dispersed in petroleum oils, then the shape and the maxima of the constituents may be altered and the positions of near neighbors reversed. This effect of the substrate, predicted years ago by E. McDonald (85), is liable

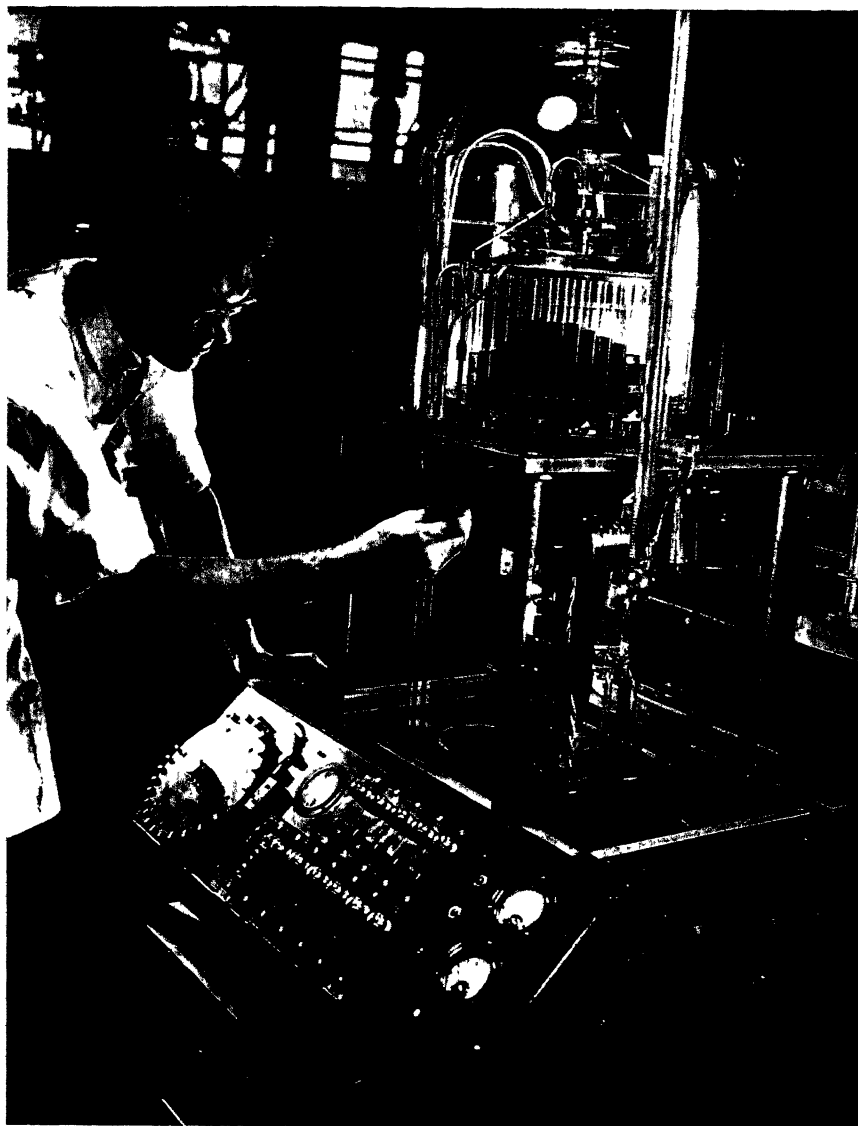


FIG. 28. Automatic, analytical, centrifugal cyclic still. Distillate tubes are seen in the crescent surrounding the evaporator.

to be misleading unless anticipated, but interpreted correctly it affords a useful clue as to the relative polarity, solubility, affinity, and thus *structure* of the solutes. Substances which can be separated no further from a glyceride sub-

strate can be transferred to a petroleum oil and the separation continued. Quantitative experiments on the influence of chemical structure on the behavior of

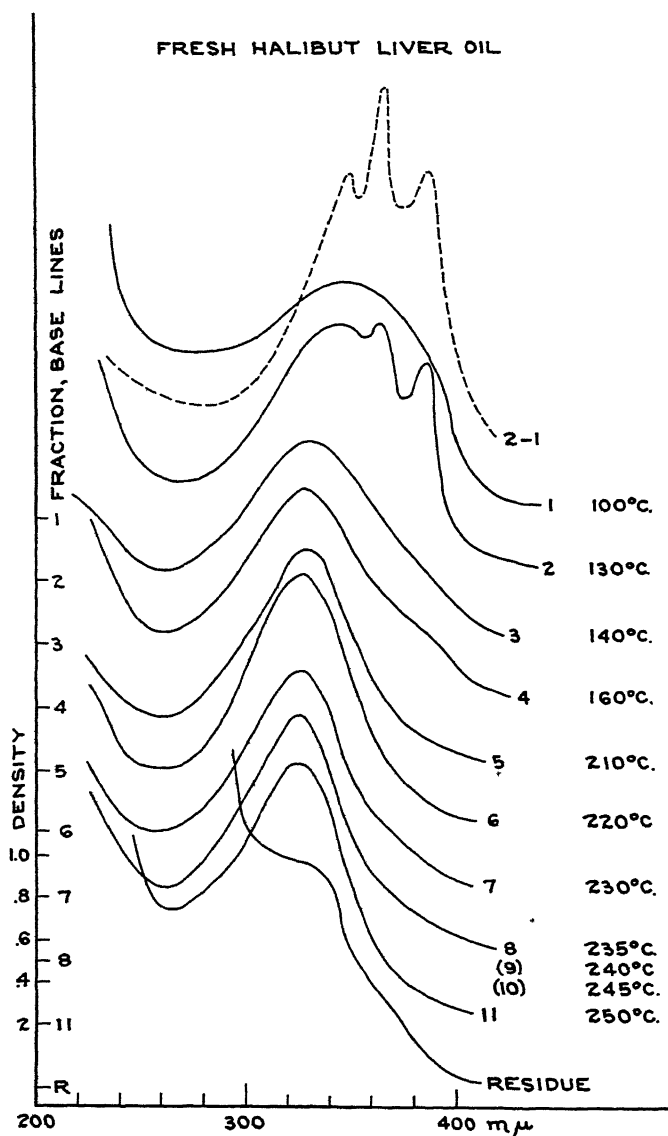


FIG. 29. Superimposed ultraviolet spectra of a succession of fish liver oil distillates

solvent and solute in the molecular still are in their infancy but should offer an approach to the fundamental study of evaporation.

The experimenter who has obtained a curve of the predicted shape remains uninformed as to its degree of nearness to the steepest possible curve corre-

sponding to the maximum separation of one theoretical plate. He must, therefore, assess the merits of the curve by comparing it with others of similar origin. Now it so happens that a good elimination curve coincides nearly with a triangle of similar area: $a = \frac{1}{2}bh$, when b and h are the dimensions of base line and height. Evidently for a fixed height the sharpness of separation is inversely proportional to the length of base line, or, in the case of the true elimination curve, of the area under the curve. When the elimination curve is plotted on graph paper, using 10°C. for one unit of base line, we find that the relation

$$\epsilon = \frac{5h}{a}$$

approximates unity in most experimental cases, where ϵ is the *elimination coefficient*, h is the height of the elimination curve, and a is the area under the curve,

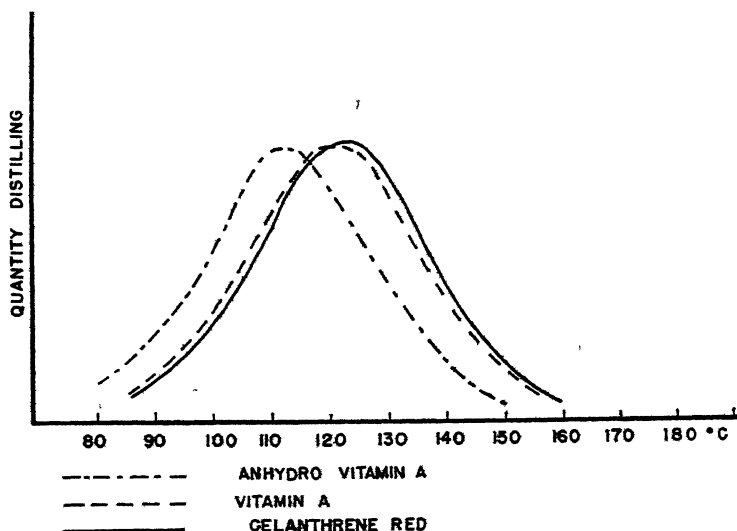


FIG. 30. Comparative elimination curves of vitamin A-like materials

the units of base line corresponding with temperature intervals of 10°C. If other temperature intervals are employed, the equation becomes

$$\epsilon = \frac{h \cdot \Delta t^{\circ}\text{C.}}{2a}$$

The elimination technique has been tested most thoroughly in relation to the fat-soluble vitamins and the diaminoanthraquinone pilot dyes. The earliest analytical distillations provided curves of predicted shape. Thus, a distillation of a non-saponifiable fraction of a fish liver oil, dissolved in glyceride controlled-yield oil, gave series of fractions which when examined in the ultraviolet spectrograph provided a set of spectra of the kind shown in figure 29. Two distinct spectral curves are discernible: the three-pronged curves typical of anhydro-vitamin A (8, 29, 93) and the simpler absorption curves, with maximum at 328

microns, of vitamin A itself. If the part of the extinction coefficient at 328 millimicrons attributable to vitamin A is plotted against fraction number, the curve shown in figure 30 results, where vitamin A alcohol is seen to have an elimination maximum at 126°C. compared with Celanthrene Red at 127°C. If the earlier fractions are combined and redistilled and the extinction coefficient at 363 millimicrons is determined for each subfraction, the elimination curve for anhydrovitamin A with maximum at 108°C. is secured. Thus, in one or two distillations it is possible to determine the relative boiling points of as many substances as there are available separate and independent tests of changes in concentration. Had the distillation of the liver oil non-saponifiable been done from a mineral constant-yield oil and residue mixture, the maxima of the constituents would have occurred in different order (data from Shantz).

SUBSTANCE	ELIMINATION MAXIMA*	
	Glyceride substrate	Petroleum substrate
	°C.	°C
Vitamin A.....	109	102
Vitamin A ₂	110	102
Anhydrovitamin A.....	90	90
Anhydrovitamin A ₂	109	108
Celanthrene Red.....	110	95

* These distillations were carried out at 5° intervals, using two cycles per temperature. Maxima obtained using one cycle at 10° intervals are about 16° higher.

A secondary effect which may distort the elimination curves is reactivity of the substance under examination at high temperature. Pilot dyes, for instance, can become chemically altered during distillation. This is particularly true with glyceride oils containing a high proportion of fatty acid. The dialkylanthraquinones are observed to exchange radicals with the fat or fatty acids, giving rise to distorted curves; the distortion, since the exchange occurs more markedly at high temperatures and long exposure, is generally observed in the high-temperature limb of the elimination curve. When this has happened it may generally be recognized, except when the exchange merely broadens the peak of the curve and thus shifts the apparent maximum upwards. Ester exchange seldom occurs in entirely neutral fats when handled quickly, and it appears to be absent when the pilot dyes are distilled from mineral oils.

During the distillation of vitamins, sterols, and other substances which can exist as esters, a sharp upturn to the distillation curve is occasionally noted at higher temperatures. Also, it is generally the higher temperature limb which shows any distortions or irregularities. When these are observed it always denotes the presence of more than one molecular species obeying the identifying test, but a careful survey should be made to find whether the extra substances were present originally or have been formed during distillation. We were unaware of this effect when examining the complexity of vitamin D (68) in cod liver

oil and our conclusions that there were extra D-vitamins of a very high boiling point and hence high molecular weight are open to suspicion. Our finding that there were low-boiling D-vitamins (8) would appear not to require revision, since interaction between a sterol and glyceride at the lower temperature has not yet been detected. A rare phenomenon, but one which would seem to be established, is a great increase in volatility due to momentary conversion to free radical. Thus, a blue condensate has been found on the fore-vacuum side of the condensation pump when using the pilot dye dipropylaminoanthraquinone which evaporates in the molecular still at about 150°C. It is unlikely that the dye would have passed through the condensation pump in any quantity unless it were momentarily disassociated.

II. APPLICATIONS OF THE ELIMINATION-CURVE TECHNIQUE

The earliest applications of the method substantiated Bacharach's (3) observation that vitamin A exists in marine oils as an ester. Two widely separated maxima were found, and it was demonstrated that the high-boiling material could be distilled entirely at the lower temperature if saponified first. Vitamin D proved to exist as a mixture of free sterol and sterol esters, though there was from 30 to 40 per cent of free material in contrast to the 0 to 5 per cent free vitamin A found in the oils. A study of the keeping qualities of various fractions of fish liver oil, coupled with measurements made by the Emmerie and Engel test (33), showed that preservative substances, later recognized by Robeson (92a) as tocopherols, distill at temperatures intermediate between the vitamin A alcohol and esters. Distillation curves of the odor, the free fatty acids, and anhydrovitamin A have been compiled, so that it is possible to present a distillation map of a typical fish liver oil showing the interrelation (52) of constituents important to the user. This is given in figure 31.

The map shown in figure 31 is more than an academic compilation. It is the foundation of an industry which has considerably augmented the supplies of vitamin A in a time of world-wide shortage. It shows that passage through the molecular still with carefully controlled rates of distillation will concentrate the natural vitamin A esters and natural preservative into a single distillate, rejecting the undesirable acids and odors in a lighter fraction and leaving the bulk of the esters of the other unsaponifiables and the parent oil undistilled and substantially unchanged. This oil, with trifling mechanical loss, is left available for food or industrial use. The process, which dates from 1930 (59), should be clearly distinguished from the distillation of vitamin A alcohol produced by saponification. In the latter case the whole of the fish liver oil must first be changed into soap, while the free vitamin A is later purified by small-scale distillation. Some of the properties of free and esterified preparations of vitamin A are listed in table 6.

When Lederer and Rosanova (83) discovered another factor in the unsaponifiable fraction of fresh water fish oils, this was soon identified as a new vitamin A (100) which resembled the old in many (43) respects, the chief difference in

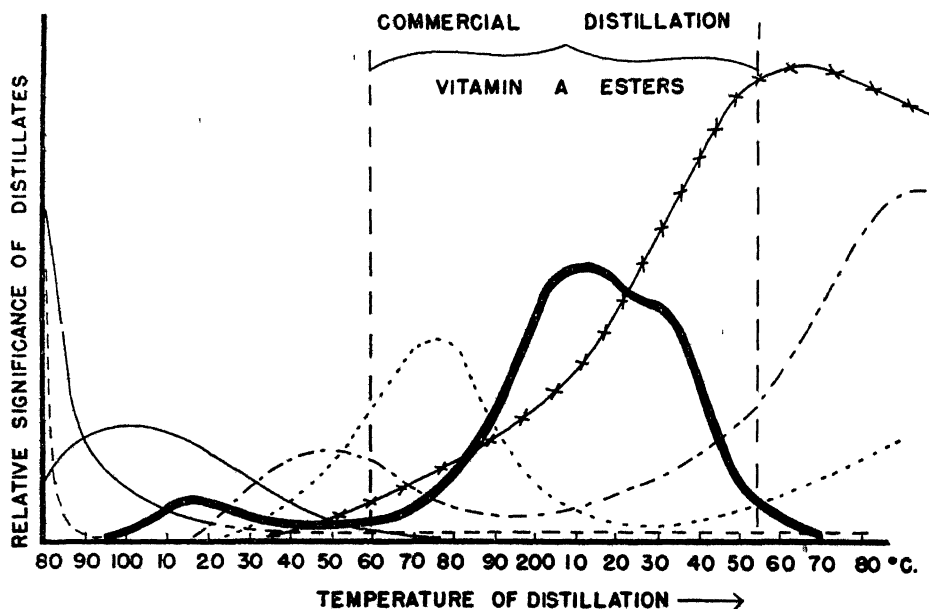


FIG. 31. Distillation map of a fish liver oil. ----, residual protein odor; - · - · - ·, rancidity and reversion odor; ———, free fatty acids; · · · · ·, sterols, vitamin D, glyceride ethers, and their esters; - - - - -, preservatives, tocopherols, and their esters; ———, vitamin A and esters; -x-x-, glyceride fat.

TABLE 6
Properties of free and of esterified preparations of vitamin A

PREPARATIONS OF VITAMIN A	ELIMINATION MAXIMUM*	AVERAGE POTENCY, U.S.P. UNITS PER GRAM (ORIGINAL)	AVERAGE POTENCY, U.S.P. UNITS PER GRAM (DILUTED FOR KEEPING TEST†)	AVERAGE CONVERSION FACTOR‡	COMPARA- TIVE STABILITY, HOURS AT 45°C.
	°C.				
U.S.P. Reference Oil II.....	214	1,700	1,700	2000	13
Halibut liver oil.....	212	120,000	120,000	2000	20-30
Distilled vitamin A ester, com- mercial concentrates.....	214	200,000	2,000	2000	200
Saponified fish liver oil, commercial concentrates.....	123	1,000,000	2,000	1500-1800	50
Crystalline vitamin A alcohol....	123	4,300,000	2,000	2460	50
Crystalline vitamin A acetate....	132	3,520,000	2,000	2350	170
Crystalline vitamin A palmitate...	208	2,310,000	2,000	2520	170

* Celanthrene Red = 126°; diamylaminoanthraquinone = 186°.

† Diluted with refined cottonseed oil.

‡ The protocols for these assays are being published in part and all are available for inspection.

constitution being the number of conjugated double bonds. Two formulae were proposed, as follows, with preference given to the second.

Gray (44) compared the elimination curves of both vitamins and found that the maxima differed by about 3°C. This seemed to disprove the theory that the vitamin chain had been lengthened by two carbon atoms. A recheck of Gray's

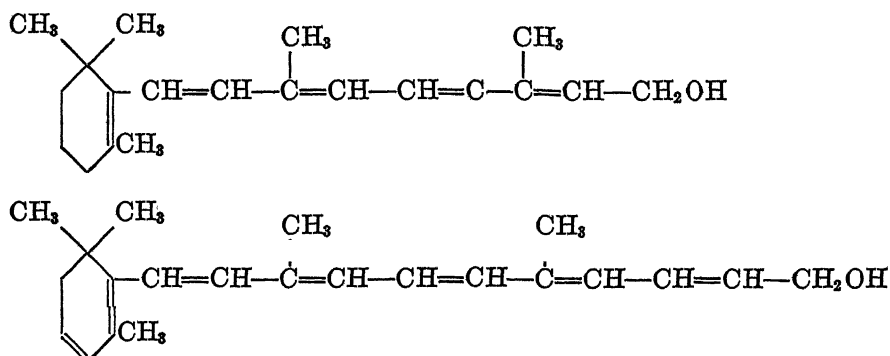


TABLE 7

Maxima of a number of saturated and unsaturated fatty acids

NAME OF ACID	ELIMINATION MAXIMUM FROM GLYCERIDE CONSTANT- YIELD OIL AT 10° INTERVALS
	°C.
Lauric.....	88.5
Myristic.....	98.0
Palmitic.....	108.0
Linoleic.....	114.0
Oleic.....	116.0
Stearic.....	118.0
9,11-Linoleic.....	123.5
α-Eleostearic.....	127.0

paper by Embree (32) showed that the difference may be much less, while Shantz and Cawley have shown the maxima to differ at most by 1°C. Gray and Cawley (45) determined the relative maxima of a number of saturated and unsaturated fatty acids (table 7) and demonstrated that the substitution of a double for a single bond shifts the maximum 1° to 3°C. The present evidence from distillation is that vitamins A and A₂ contain the same number of carbon atoms.

Analytical distillation has found an interesting application as yet only partly explored,—investigating the metabolism of those vitamins and sterols which can exist in more than one state of combination. Thus, cholesterol taken from the animal body is found to be a mixture of free and esterified sterol, the shape of the complex elimination curve allowing both the proportions and kinds of the esters to be deduced. Vitamin A is another case in point which has been investigated by Gray and others (47). The vitamin occurs naturally as the carotenoids, as

various esters in butter and marine oils, and as free vitamin A. The questions arose: Do all these substances become one substance or a similar mixture of substances after ingestion? Or is each stored and utilized in the form swallowed? Or is the state of the metabolized vitamin dependent on the species of animal? To investigate the matter six different forms of vitamin A were fed to six groups of young rats. The animals were killed, the livers extracted, the extracts blended with glyceride controlled-yield and residue oil, and distilled. The fractions were tested with antimony trichloride and the extinctions of the blue colors were

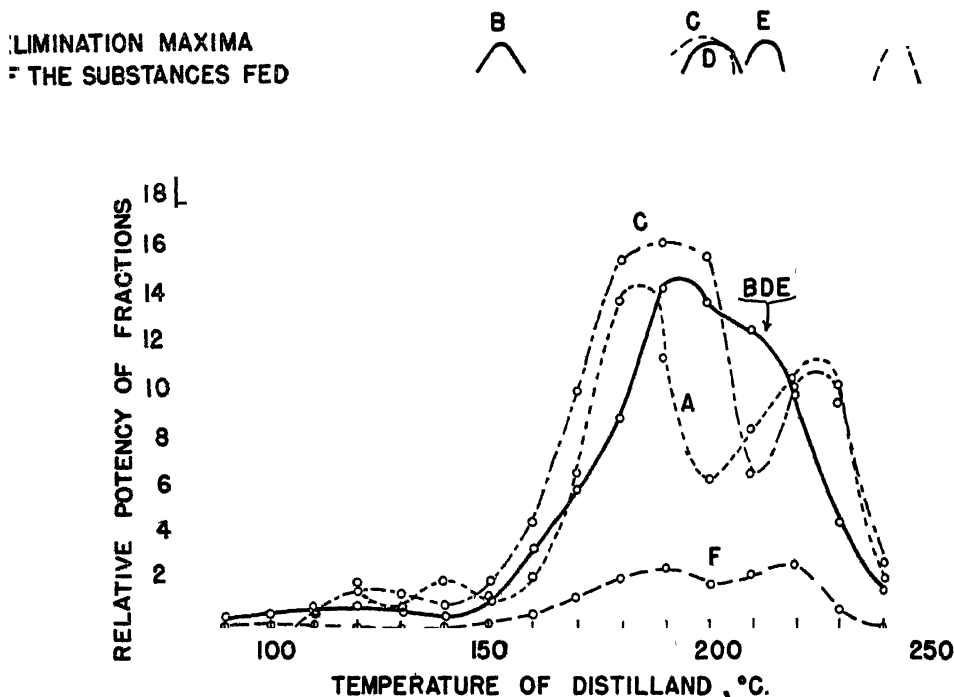


FIG. 32. Distillation map showing condition of various vitamin A compounds after ingestion by rat and extraction from liver. A (---), vitamin A-alcohol, 39 per cent recovery; BDE (—), vitamin A stearate, vitamin A caproate, and distilled ester concentrate, 44.2-50.7 per cent recovery; C(---), U.S.P. Reference Oil I, 55.7 per cent recovery; F(—), β -carotene, 2.7 per cent recovery.

plotted against temperature, as in figure 32. The antimony trichloride measured impartially the free vitamin A, the vitamin esters, anhydrovitamin A, and other vitamin A products. It was established that no matter what form of vitamin was fed there appeared in the rat's liver a few hours later a preponderance of vitamin A ester with an elimination maximum corresponding to palmitate (185°C.), a smaller quantity of higher molecular weight ester (230°C.), a still smaller quantity of free vitamin A (125°C.) with occasional traces of anhydrovitamin A (105°C.), and another vitamin A derivative

(115°C.), both probably produced during the alcohol extraction of the livers (Cawley, 1942). Gray has since found (46) that when the rats are kept alive for some weeks after administration, the heavy ester is absent from the liver, most of the vitamin having been converted to palmitate. We quote these experiments to show that considerable information can be extracted from the relatively poor separating power of the molecular still.

The elimination technique matured at a time when the multiple nature of vitamin D occupied particular attention. Calciferol had been differentiated from fish liver oil vitamin D by the rat-chicken ratio test of Bills and Massengale (87); Bills (7) had listed at least eight substances of vitamin D activity, while Windaus (110) and Brockmann (9) were arguing to the contrary that irradiated 7-dehydrocholesterol was the only important antirachitic substance in tuna and other fish liver oils. It seemed worthwhile to examine the homogeneity of marine vitamin D by molecular distillation. In doing this, Hickman and Gray (68) were confronted with the difficulty that no sensitive chemical test existed for the vitamin, forcing them to use the inexact method of rat assay. Each point on the elimination curve was thus the tombstone of a score of rats, the complete curve marking a cemetery of no mean proportions. The conclusion of some years of work,—namely, that there are at least four and possibly six D vitamins in Norwegian cod liver oil (*Gadus morrhua*),—is still equivocal but the method of approach deserves mention.

The cod liver oil was saponified to destroy vitamin esters and then blended with constant-yield oil and distilled. The highest potency fraction was sought by pilot assay, and assay levels were festooned from this point to cover the probable potencies of the other fractions. There resulted a complex curve which could lie within the ambit of a single curve and might, therefore, be considered merely a measure of the imperfection of the assay were it not that repeated distillations gave substantially the same complex curve. An average of three curves is recorded in figure 33, where the dark central line shows the probable potencies of the fractions and the faint lines show the outside credible limits of potency. The question at issue became: Are the multiple peaks and inversions real or are they—to beg the question—artifacts?

A master distillation was performed to provide in any fraction enough vitamin D to form the starting material for a subdistillation after reblending with constant-yield oil. Fractions coming over between 100° and 130°C. were combined and redistilled as were fractions between 160° and 200°C. If one vitamin only were involved, the secondary distillation curves should each resemble the primary, but if the primary low- and high-boiling fractions contained separate antirachitic substances, second distillations should furnish widely different curves. Figure 34, where the second distillations are superimposed, shows that the difference indeed exists, each curve substantiating the existence of real antirachitic maxima in its own temperature region. The lesson from this experiment is that when the shape of a distillation curve is in doubt after repeated experiment, the contours can be proved or disproved by redistillation of the high and low fractions.

Since the beginning of interest in marine liver oils the spectrograph has revealed many anomalies in the ultraviolet and antimony trichloride absorption curves

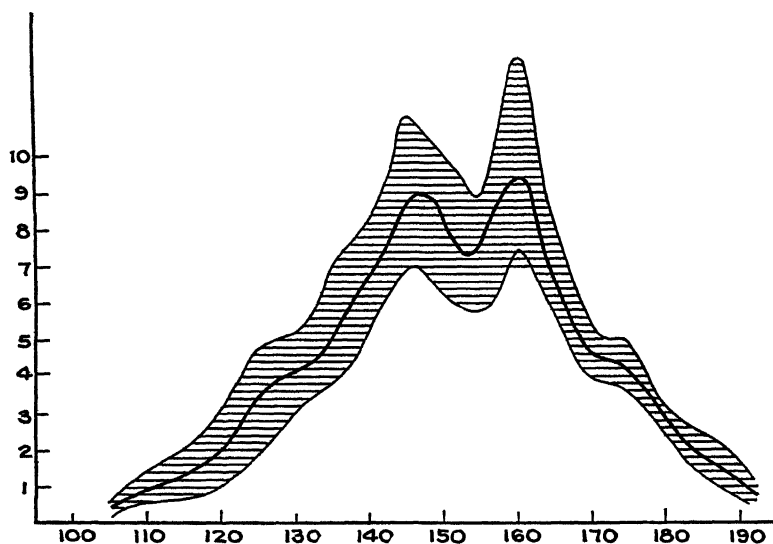


FIG. 33. Averaged elimination curve of the saponified vitamin D from whole cod liver oil

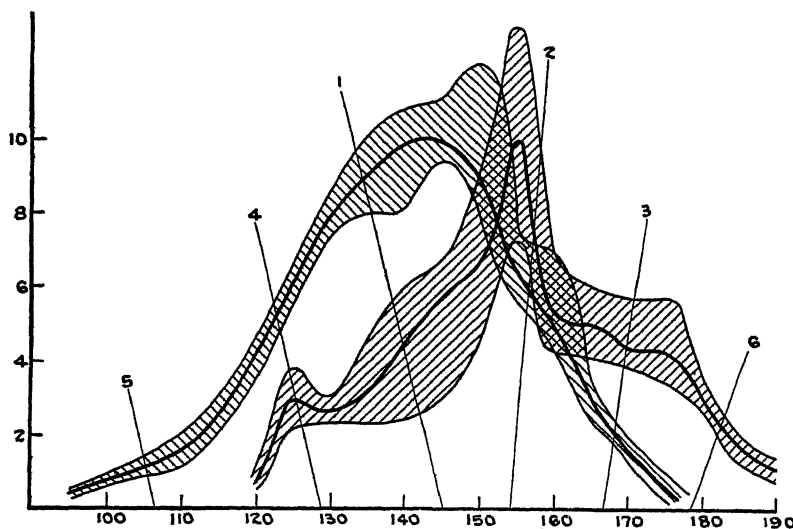


FIG. 34. Superimposed elimination curves of "low-boiling" and "high-boiling" vitamin D distillates. Note that in spite of irregularities of assay the curves fail to overlap except in their central portions, thus demonstrating separation of two varieties of natural vitamin D.

attributed to vitamin A. The abnormalities are gradually being identified with other substances present in the oils. Anhydrovitamin A has been concentrated

TABLE 8
Substances investigated quantitatively by means of the molecular still†*

VITAMIN A CLASS	VITAMIN D CLASS	STEROL CLASS	HORMONE CLASS	DYE CLASS
Vitamin A alcohol* (52) Vitamin A acetate* (47) Vitamin A palmitate* (47) Vitamin A naphthoate Vitamin A acid succinate* (95) Vitamin A succinate* (95) Vitamin A fish liver esters* (52) Vitamin A rat liver esters* (47) Vitamin A ₂ * (44) Anhydrovitamin A* (93) Sub-vitamin A* (99) 1-Carotene* (95) 1-Carotene (95) Xanthophyll (95) Xitol* (114)	D ₂ calciferol* (68) D ₃ * (68; Windaus) Cod liver oil D* (68) Tuna liver oil D* (68) Swordfish D* (68)	Cholesterol* (54) Sitosterols* (54) Stigmasterol* (54) α-Tocopherol* (54) γ-Tocopherol Tocoquinones Vitamin K	Progesterone (1) Bone marrow ane- mia factor (74)	Substituted diaminoan- thraquinones* (52) Indigo and derivatives* (52) Butter coloring matters (95)

* Only substances marked with asterisks have been examined quantitatively. The elimination maxima are not given because the figures do not yet give a concordant series.

† This list does not include the many investigations of the natural glycerides and heavy petroleum residues.

by distillation to the point where Robeson and Baxter have obtained it in crystals, m.p. 74–75°C., and Embree and Shantz have been able to determine its probable

TABLE 9*
Crystalline vitamin A

VITAMIN A PREPARATIONS	MELTING POINT	BIOLOGICAL POTENCY†	CONVERSION FACTOR BIOLOGICAL POTENCY		
			$E_{1\text{ cm.}}^{12}$ (328 m μ)	$E_{1\text{ cm.}}^{12}$ (528 m μ)	$E_{1\text{ cm.}}^{12}$ (620 m μ)
	°C.	U.S.P. units per gram $\times 10^{-6}$			
Vitamin A alcohol.....	63–64	4.3	1750	2460	4800
Vitamin A acetate.....	57–58	3.52	1510	2350	4580
Vitamin A palmitate.....	27–28	2.81	940	2520	2535
Vitamin A succinate (di).....	76–77	3.14	1240	2630	4450
Vitamin A β -naphthoate.....	74–75	3.44	1090	3160	2940
Anhydrovitamin A.....	76–77	0.015	3650‡	4.1	5500
Vitamin A β -naphthoate§.....	78	2.22	1190	1870	
Vitamin A anthraquinone-2-carboxylate (yellow form)§.....	126	1.75	938	1870	
Vitamin A anthraquinone-2-carboxylate (red form)§.....	118–120		1090		

Vitamin E

	MELTING POINT	WAVE LENGTH	$E_{1\text{ cm.}}^{12}$	POTENCY¶ RELATIVE DOSE REQUIRED
	°C.			
Natural α -tocopherol.....		292	73.7	0.8
Natural β -tocopherol.....		297	87.6	1.5
Natural γ -tocopherol.....		298	92.8	8.0
Merck synthetic α -tocopherol.....		292	75	1.0
α -Tocopherol succinate.....	76 – 77	286	37	0.68
α -Tocopherol palmitate.....	42 – 43	286	26.8	
α -Tocopherol acetate.....	26.5– 27.5	285	41.2	
α -Tocopherol allopnanate.....	158 –160	286	36	
γ -Tocopherol palmitate.....	44 – 45	286	40.7	8.0
γ -Tocopherol allopnanate.....	137 –139	286	46.3	
β -Tocopherol azobenzenecarboxylate.....	69.5– 70.5	328	483	6.0
β -Tocopherol allopnanate.....	135 –137	283	44.5	

* Data assembled and checked by Dr. J. G. Baxter.

† Assays by Dr. P. L. Harris using U.S.P. Reference Oil II.

‡ Value at 371 m μ .

§ Values obtained by Mead, Underhill, and Coward. β -Carotene used as standard for biological assay.

¶ By Dr. P. L. Harris using Mason-Bacharach rat test. Values represent median fertility dose as milligram of free tocopherols.

constitution. These workers have also studied "sub-vitamin A" (32) by a combination of distillation and chromatography. Perhaps the most interesting

marine material examined by Embree and Shantz (32a) is a substance, previously recognized by its ultraviolet absorption but otherwise uninvestigated, which occurs in whale liver oil. The substance, tentatively named "kitol", has an absorption band at 285 millimicrons and the antimony trichloride product has a band at 428 millimicrons. When heated to 250°C. kitol disappears, giving rise to vitamin A. The total quantity of vitamin A in whale liver oil is thus *increased* by distillation. Kitol must take its place as a new kind of provitamin A and as the only vitamin A precursor which has been converted to the vitamin *in vitro*. It differs from other known precursors by having no vitamin A activity until heated. Embree and Shantz recently report finding the equivalent precursor of vitamin A₂ in pike liver oil. Both provitamins were discovered by means of *quantitative* molecular distillation.

Some of the substances which have been investigated in the analytical still are listed in table 8. The properties of various vitamin and related substances which have been crystallized by Baxter and Robeson are given, by their kind permission, in table 9. Further applications of the molecular still and the elucidation of the structure of marine glyceride oils are described by Farmer and van den Heuvel (35). A general summary by Embree (30) has appeared in this journal.

III. QUALITATIVE LABORATORY DISTILLATIONS

*The elimination technique supplies information rather than actual separation. When the boiling range of the wanted constituent has been learned, larger scale distillation can be done with or without pilot dye, collecting few distillates at the preferred temperatures. Distillation seldom yields a pure product from a crude mixture, only an increase in concentration. This increase, however, may be sufficient to allow the material to be separated by means which were previously ineffective. Materials which could not be crystallized from the crude may crystallize readily from the richer fractions.

IV. FUTURE OF THE METHOD

The simple laboratory still is reaching a stage of development where further great increase in efficiency is unlikely. But even when finality of design is neared, there will remain unexplored the larger and more important field of high-vacuum fractionation. A still with greater fractionating power than one theoretical plate—two would be a signal advance—will extend the method appreciably. We are only just consolidating our knowledge of how to make the molecule take a single leap into empty space from a very thin film of distilland. It remains to train the same molecule to dance precisely through a succession of evaporators and condensers until it loses all competitors and emerges only in the company of its fellows.

The sources of the historical material used have been quoted in the introduction. It remains to pay grateful tribute to the owners of Distillation Products and members of the staff whose support and efforts have made possible the in-

tegrated development of the high-vacuum distillation process and its organized application to research. Mr. George Surridge has been responsible for the machine drawings and much of the design; Mr. F. M. Jenner has superintended the construction of large apparatus; Dr. J. Hecker has brought the apparatus into operation and over a period of years greatly influenced the design. Dr. N. Embree has led in the application of the stills in pure research; his colleagues have been mentioned in the text.

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THE CHEMISTRY OF TUBERCULIN

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Tuberculin to the bacteriologist is the concentrated bacteria-free liquid culture in which the tubercle bacilli have grown. To the physician it is a product of the tubercle bacillus which gives a specific reaction in an individual who has at some time been infected with the organism. To the chemist it signifies the particular ingredient of this culture medium which is responsible for the specific reaction, and this has come to mean protein (34); hence this fraction will receive most consideration in this article.

There are, however, other constituents of tuberculin which are of considerable interest to the chemist, in spite of the fact that they have not so far proved to be of great biological significance. For example, one finds, in addition to the protein, considerable quantities of polysaccharide and nucleic acid in a culture filtrate of the tubercle bacillus grown on a synthetic medium such as Long's (18). In fact, these two colloidal constituents exist in such significant amounts and in such combination with the protein that they become the chief impurities which are most difficult to remove from the protein during its purification. For this reason considerable research has been done upon these fractions and consequently much information is available concerning their chemical properties.

I. CARBOHYDRATE OF TUBERCULIN

The carbohydrate in tuberculin is in the form of polysaccharide. It exists chiefly as molecules sufficiently large that they do not pass through thick collo-dion membranes; however, some do pass the finest guncotton membranes used (37). Evidence has been presented to indicate that much of the polysaccharide

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is adsorbed to the tuberculin protein molecules, which it accompanies through many precipitations and from which most of it can be released by bringing the protein repeatedly to its isoelectric point. As much as 50 per cent of the colloidal content of raw tuberculin may consist of polysaccharide.

A. Composition of tuberculin polysaccharides

Polysaccharide isolated from the culture medium has been shown on hydrolysis by Renfrew (28), Dorset and Henley (7), and Heidelberger and Menzel (12) to consist of *d*-arabinose, *d*-mannose, and *d*-galactose. Watson (48) has detected also a small amount of glucosamine.

The polysaccharide can be determined quantitatively (26) after hydrolysis, which involves preliminary digestion with 3 per cent sulfuric acid for 7 hr., by means of the Shaffer-Hartmann microcuprous method or the Hagedorn-Jensen method. The latter method, however, yields more accurate results, since it will detect pentose as well as glucose, and consequently gives 95.3 per cent reducing substances, calculated as glucose, in pure tuberculin polysaccharide, whereas only 43.9 per cent glucose is found by means of the Shaffer-Hartmann method.

A simpler method, which does not involve a long period of preliminary digestion, is the carbazole reaction (6); this has been used almost exclusively by the author in recent years (33) with an adaptation to the Evelyn colorimeter.

As will be shown later, it is extremely difficult to remove all traces of nucleic acid and protein from the polysaccharide, but it seems reasonable, in view of the data at hand, to consider the nitrogenous constituents as impurities, at least of a certain portion of the polysaccharide.

B. Physicochemical properties of the polysaccharides

When studied in electrophoresis by means of the Tiselius technic (47), the tuberculin polysaccharide from the human-type tubercle bacillus was found to possess no, or very little, mobility; consequently it could be separated easily from the protein in raw tuberculin by allowing the latter and other impurities to migrate away from it (40). In this way a small amount of a product was isolated which proved by analysis to be 90 per cent polysaccharide. This product, when studied in the ultracentrifuge, was found to have the same sedimentation constant, S_{20} about $1.8S$ (one Svedberg unit, $S_1 = 1 \times 10^{-13}$ c.g.s. units), as a polysaccharide which had been chemically isolated from raw tuberculin and which contained only 0.09 per cent nitrogen. There was no drift in the value of the sedimentation constant with change in concentration. The diffusion constant, D_{20} , was 11.0×10^{-7} ; the specific volume was 0.619 at 20°C.; and a molecular weight of 9000 was calculated, with a molar frictional ratio, f/f_0 , equal to 1.5. These polysaccharides showed a remarkable degree of homogeneity, in contrast to most polysaccharides hitherto studied, especially those of other bacteria.

Later Tennent and Watson (46) isolated by means of electrophoresis polysaccharides from the culture filtrates of the human-, avian-, and bovine-type tubercle bacilli and of the leprosy bacillus and *B. phlei*, and studied their molecular-kinetic properties and serological specificities. The molecular weights of

the polysaccharides from the human-type tubercle bacillus and the *B. phlei* filtrates were found to be 7300, that from the avian-type tubercle bacillus filtrate was 7200, and that from the leprosy bacillus filtrate was 2500. The dissymmetry numbers and the ratios of major to minor axis indicated that none of the polysaccharides was greatly elongated, and that they probably have a closed rigid structure.

A simple scheme for separating the polysaccharide from the protein and nucleic acid on a large scale by means of electrophoresis has been outlined by Seibert and Watson (41). By this method two types of polysaccharide were isolated from a large quantity of heated tuberculin filtrate. One polysaccharide was colorless, contained only about 0.2 per cent nitrogen, and did not migrate in the electrical field. The other, present in much larger quantity, had a low mobility, and the nitrogenous impurity could not be removed to less than 0.85 per cent nitrogen, even by electrophoresis for 114 hr. in phosphate buffer at pH 7.3, $\mu = 0.02$, and a potential gradient of about 3.7 volts per centimeter.

These two types of polysaccharides were studied comprehensively by Watson (48), who found the immobile form of polysaccharide which contained less nitrogen to be less easily hydrolyzed and to have a lower content of reducing sugar, of nucleic acid, and of glucosamine, a higher neutral equivalent, a higher positive rotation, a lower molecular weight, and a more spherical form than the one with low mobility. He concluded that these two polysaccharides corresponded to many of the fractions with similar properties which were isolated by Heidelberger and Menzel (12) from the bodies of the tubercle bacilli.

C. Biological properties of the polysaccharides

All of these polysaccharides specifically precipitated the serum of animals immunized against the tubercle bacillus, as has been shown by Laidlaw and Dudley (15), Mueller (25), Renfrew (28), Masucci, McAlpine, and Glenn (21), Seibert, Pedersen, and Tiselius (40), and Tennent and Watson (46). The last two investigators concluded that the polysaccharides were genus-specific rather than species-specific. Heidelberger and Menzel (12) and Karjala and Heidelberger (14) had also previously been unable to demonstrate that the human or avian tubercle bacillus polysaccharides were species-specific.

Enders (9) claimed that he was able to produce uterine strip contraction as well as typical lethal anaphylactic shock with the polysaccharide isolated by Mueller (25) in guinea pigs actively sensitized with dead tubercle bacilli or passively sensitized with the serum of immunized rabbits, but since his preparation contained 0.3 per cent nitrogen it can be questioned whether the polysaccharide itself was responsible for the reactions. The polysaccharide has appeared to resemble a haptene rather than an antigen, since antibody production did not result from repeated injection of the polysaccharide or polysaccharide-containing fractions, while a high precipitin titer could be obtained when it was added to immune sera (12, 32).

The work of Sabin, Joyner, and Smithburn (29) showed that the polysaccharides of the tubercle bacillus exerted a chemotactic effect on neutrophilic

polymorphonuclear leucocytes. These cells were immediately accumulated at the local site of injection, and soon after intraperitoneal injection young polymorphonuclear leucocytes appeared in the blood stream. No general toxicity resulted, a result which has been confirmed by the author with the polysaccharides from tuberculin.

Cournand and Lester (5) stated that a polysaccharide fraction isolated from the tubercle bacillus by Heidelberger and Menzel (12), when injected intracutaneously into tuberculous human beings, elicited both a distinct early and a delayed type of reaction. Since their polysaccharide was known to contain nitrogen, which might represent contaminating tuberculin protein, they treated their preparation with trypsin and still were able to obtain the early type, whereas the delayed type of reaction similar to that obtained by means of the tuberculin protein was destroyed. This they concluded was evidence that the polysaccharide itself was responsible for causing the early type of reaction.

McCarter and Watson (22) reinvestigated this conclusion and found that the undenatured form of tuberculin protein caused reactions reaching their maximum at 6 hr., as well as the typical delayed type of tuberculin reaction, and that the tuberculin protein when digested with trypsin, even to the extent that it was no longer precipitable by trichloroacetic acid, still possessed considerable ability to cause the early type of reaction. Moreover, weak doses of unhydrolyzed protein injected into sensitive individuals, or strong doses into less sensitive individuals, gave reactions which were definite at 6 hr. and faded out in 48 hr. The loss in activity, under the effect of trypsin, of the protein contained in the polysaccharide was much slower than it was in the isolated protein. This suggested that the combination of the two affords a certain degree of protection to the protein. Moreover, it is evident from the work of Linterstrøm-Lang *et al.* (17) that native proteins are less readily attacked by proteolytic enzymes than denatured proteins.

The strongest evidence of McCarter and Watson that the protein in the polysaccharide was the cause of the early-type reaction lay in the results obtained in an experiment on mild acid hydrolysis. The concentration of acid and conditions of hydrolysis were determined which were sufficient to hydrolyze the polysaccharide completely, as followed by a determination of the per cent of reducing sugar found with increasing time of hydrolysis. It was then noted that under these conditions, even though the polysaccharide was completely hydrolyzed, its ability to cause an early type of reaction was never completely destroyed, just as was true in the case of the pure protein when similarly treated. These results make it probable that the polysaccharide *per se* is not responsible for a significant specific local skin reaction.

II. NUCLEIC ACID OF TUBERCULIN

Nucleic acid is the other colloidal constituent which is found usually accompanying the protein. Its concentration in various protein fractions may vary from zero to 30 or 40 per cent, depending upon the source and the mode of preparing the protein. For example, the preparations that are made from tubercu-

lin obtained from heated cultures usually contain more nucleic acid than those protein fractions precipitated directly from unheated culture filtrates, and there is evidence that at least a portion of the protein and nucleic acid in these heated preparations may exist as nucleoprotein, which travels as a single component in electrophoresis at all hydrogen-ion concentrations.

Much of the nucleic acid is bound in a looser combination to the protein, since at a pH of 5.0 or at a more alkaline pH the two dissociate, whereas at more acid reactions they travel as a single component (33). It was pointed out that, in view of the fact that the dissociation constants of the imino group of histidine in protein and also of the secondary phosphoric acid group in nucleic acid are both in this region, it is probable that the link between the protein and the nucleic acid may occur through these two groups. Complexes between nucleic acid and serum albumin have been described by Stenhagen and Teorell (44), and between nucleic acid and ovalbumin by Longworth and MacInnes (20).

Recognition of this type of combination led to a practical method for removing nucleic acid from the protein during the purification of the latter. For example, in the preparation of a large quantity of purified tuberculin protein, which was known as *Purified Protein Derivative* (36) and was to be used as a standard in tuberculin testing, the final purification was made by repeated precipitation with ammonium sulfate at a pH of 7.0, and the resulting product contained only 1.2 per cent of nucleic acid.

A. Composition and quantitative determination

All of the nucleic acid found in tuberculin appears to be of the desoxyribose type and can be measured quantitatively by means of the diphenylamine reaction (40), whether it is free or in combination with protein. A modification of the original method given by Dische (6), utilizing the Evelyn colorimeter, has been outlined by Seibert (33).

B. Physicochemical properties

By analysis it was found (40) that the component in tuberculin migrating with the greatest mobility in electrophoresis was nucleic acid. When free it traveled at practically the same rate as the thymus nucleic acid isolated by Hammarsten (11). In the case of both nucleic acids the main gradient exhibited a sharp and distinct boundary when the migration proceeded into buffer, whereas there was considerable spreading when the migration was into the solution. Moreover, very small gradients with lower mobility continually separated from both nucleic acids during their migration. These were considered to be small amounts of protein which gradually separated from the nucleic acid, since analyses had indicated there was approximately 4.7 per cent of protein present in the tuberculin nucleic acid, and 1.5 per cent in the Hammarsten preparation. It was interesting that with the separation of these components from the latter preparation the mobility² of the nucleic acid in phosphate buffer at pH 7.3 and $\mu = 0.02$ gradually increased from -17.5 to -23.5 to -26.1×10^{-5} . This effect of the

² All electrophoretic mobilities are recorded in this paper in units of $\text{cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$.

presence of protein in lowering the mobility of nucleic acid has been observed to be true also in the case of the tuberculin nucleic acid. The fastest component of tuberculin preparations has been found to have, with varying concentrations of protein and nucleic acid and under the same conditions of pH, ionic concentrations of buffer, and potential gradient, a wide range of mobility, but always lower than that found for the purest nucleic acid so far studied (-23.6×10^{-5} on the descending side at pH 7.3, $\mu = 0.02$, and a potential gradient of 6.5 volts per centimeter) (41). This same effect has been noted by Stenhagen and Teorell (44) in mixtures of serum albumin and thymonucleic acid.

The viscosity of the nucleic acid isolated from tuberculin is very much less than that of the thymonucleic acid of Hammarsten, indicating that its molecular weight may also be much less than the 500,000 to 1,000,000 determined by Signer, Caspersson, and Hammarsten (42) for the latter preparation.

The present war has interrupted further studies of the tuberculin nucleic acid which were planned by Professor A. Tiselius of Uppsala.

C. Biological properties of the nucleic acid

No obvious reaction is elicited in tuberculous animals by intracutaneous or intraperitoneal injection of the nucleic acid. It has not proved to be antigenic or to precipitate antisera to the tubercle bacillus. So far, therefore, no important biological function has been found for the nucleic acid. Indirectly, through its avidity for the tuberculin protein, there may be ascribed to it a rôle as modifier of the properties of the protein.

III. PROTEIN OF TUBERCULIN

When the polysaccharide and nucleic acid are removed almost completely from tuberculin by mild methods, as indicated above, there remains a very complex protein solution. It can be demonstrated that in this solution there are proteins which differ as to solubility, coagulability, diffusibility, sedimentability, electrophoretic mobility, antigenicity, and potency.

A. Types and properties of proteins present in tuberculin

It has become evident that raw tuberculin contains at least two proteins, with somewhat definite properties, which may be considered as undenatured proteins. They have been named proteins A and B.

Both of these proteins have been found to be soluble at all hydrogen-ion concentrations, and they have been distinguished so far chiefly by the fact that in phosphate buffer at pH 7.7 and $\mu = 0.1$ the A protein has a lower mobility (-3.3 to -4.4×10^{-5}) than the B (-6.3 to -6.7×10^{-5}) in electrophoresis. Much more at the present time can be said about the A protein than about the B, since the latter has only recently been recognized.

The pH-mobility curves of the A and B proteins have been studied in a solution containing a mixture of the two (see figure 1). It is significant that the chief difference found between these proteins was that the B protein contains more polar groups whose dissociation constant is in the neighborhood of pH 5.0—

6.0 and may, therefore, be imidazole groups. It is highly probable that it is one of these two soluble proteins which was found to crystallize (30).

Other properties which have been noted for the A protein are that it is coagulable by heat, does not diffuse through thick guncotton or cellophane membranes, and has a molecular weight of about 32,000 (40). For specific data see table 1. It exists in largest quantity in tuberculin preparations which have not been

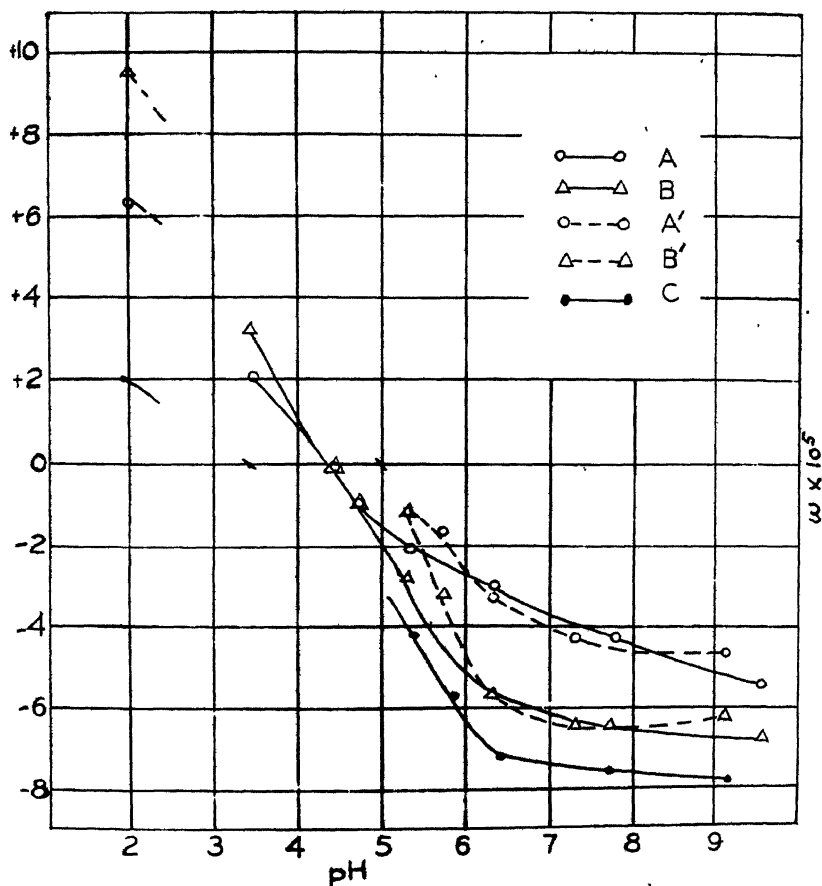


FIG. 1. pH-mobility curves. A and B were soluble proteins of TPU-84. A' and B' were proteins precipitated from PPD-S at pH 4.7. C was protein TPT-18E(A).

heated at all, or heated only mildly, and is considered to be a highly antigenic form of tuberculin protein. The properties of the B protein have not yet been studied, since it has only recently been recognized.

Recent experiments are showing that at pH 4 to 5 there can be precipitated, especially from heated tuberculins, fractions which show two protein components with approximately the same electrophoretic mobilities as proteins A and B.

These have been called A' and B'³ and may be considered as denatured forms of the A and B proteins which have lost their solubilities in the region of the isoelectric point.

Moreover, these two proteins, A' and B', differ from the A and B proteins in a shift to slightly more alkaline isoelectric points, as can be seen in the pH-mobility curves in figure 1. This corresponds to the findings with other denatured proteins (1, 27). With the exception of this shift in the isoelectric point, the curves do not differ greatly in character from those of the undenatured proteins. Slight differences can not be considered significant, since errors may be introduced in the determination of mobilities in mixtures where components are not well resolved, as was the case in these experiments.

These A' and B' proteins were obtained by precipitating the preparation PPD-S (36) at pH 4.7, redissolving the precipitate in phosphate buffer at pH

TABLE 1
Molecular properties of tuberculin proteins

PROTEIN FRACTION	PRECIPITATES AT pH	SEDIMENTATION CONSTANT S_{20} (S units)	SPECIFIC VOLUME	DIFFUSION CONSTANT $D_{20} \times 10^7$	MOLECULAR WEIGHT	MOLAR FRICTIONAL RATIO f/f_0	ELECTROPHORETIC MOBILITY* $\mu \times 10^6$
A . . .	Soluble	3.3	0.700	8.2	32,000	1.2	3.3 to 4.4
A' . . .	4.3 to 4.7 (?)						3.5 to 4.6
B . . .	Soluble						6.3 to 6.7
B' . . .	4.3 to 5.0	1.2	0.739	6.7	16,000	1.9	6.5 to 7.3
C . .	4.3 to 5.0	Heterogeneous		Heterogeneous			7.6 to 8.7
D . . .	4.3 to 5.0	Heterogeneous		Heterogeneous			5.1 to 5.6

* In phosphate buffer, at pH 7.7, $\mu = 0.1$, and a potential gradient of 9 to 10 volts per centimeter.

7.7, and reprecipitating nine times at pH 4.7 with acetic acid, using the glass electrode.

Since it was only recently, mainly through these experiments, that the apparent existence of the denatured form of the A protein—namely, the A' protein—was recognized, its further properties have not yet been determined.

The B' protein has been recognized for some time and therefore studied to some extent. It was found not to be coagulable by heat and to have a molecular weight of about 16,000 (40); its molar frictional ratio of 1.9 indicates that it is elongated, with about an 18-to-1 ratio for the major to the minor axis. Probably this indicates uncoiling of the native molecule, which occurs in the early stages of denaturation. This fraction has been shown to be non-antigenic but potent as a tuberculin.

In addition to these native and denatured proteins a whole series of molecules

³ The B' protein in this paper corresponds to the B protein noted in a previous paper (39).

with intermediate or higher mobilities and lowered tuberculin potency may be found, especially in heated tuberculin. They also precipitate at pH 4 to 5 and consequently make the separation of B' protein difficult.

The data assembled indicate that some of these molecules, which have been called D proteins, may represent extensively denatured proteins which may have been formed by the polymerization of small degraded units of the proteins, resulting in chains of many different lengths and consequently many different molecular sizes. Ultracentrifuge evidence for the existence of such molecules was presented in a previous paper, dealing with fraction TPA-1a (39). The electrophoretic diagram of this fraction at pH 7.7 and $\mu = 0.1$ showed the presence of a chief component with a mobility -5.1×10^{-5} , intermediate between A and B proteins, and two very small ones with mobilities -3.7×10^{-5} and -7.6×10^{-5} . Another recently isolated fraction, Q(E), contained a single electrochemical component with a mobility of -5.6×10^{-5} , and has been reported by Mrs. Bevilacqua in Dr. J. W. Williams' laboratory to be extremely inhomogeneous in sedimentation and diffusion.

TABLE 2
Molecular properties of some tuberculin protein fractions

TUBERCULIN	SEDIMENTATION CONSTANT S_{20} (S units)	DIFFUSION CONSTANT $D_{20} \times 10^7$	MOLECULAR WEIGHT	SVEDBERG DISSYMMETRY NUMBER f/f_0	RATIO OF MAJOR TO MINOR AXIS a/b
69, avian	1.34	3.38	36,800	2.85	40.0
69-2, avian	1.20	8.21	13,600	1.64	11.8
67, human	0.62	6.71	8,500	2.33	29.6
67-2, human	0.99	14.64	6,300	1.19	4.1

Pedersen (27) noticed a similar polydisperse sedimentation but electrophoretic homogeneity of denatured serum albumin. If one accepts the idea of polymerization of degraded units, the fact of electrophoretic homogeneity can be explained on the principle established by Abramson (1),—namely, that regardless of the size, particles of similar material travel with the same velocity.

Watson (22, 48) has given impressive evidence that precipitation at pH 4 to 5 does precipitate the more elongated or denatured forms of the tuberculin protein. Table 2, containing data from his studies, shows that the fractions which were precipitated at pH 4.3 from both an avian-69 and a human-67 tubercle bacillus-type protein contained larger molecules than the soluble fractions, 69-2 and 67-2, remaining in the filtrates. The noteworthy fact, however, is that in both cases these larger molecules were also much more elongated than the soluble protein fractions, indicating more extensive unleafing of the molecule and even polymerization. This fact was true even when the molecules were considerably degraded in size by heat, as in the case of the 67 fractions listed.

In view of these facts, it must be clear that a molecule, like 69 in table 2 with a weight of about 36,800, probably due to polymerization by elongation, may have

very different properties from those of a globular molecule such as the A protein mentioned above which has a similar molecular weight, 32,000.

Still other protein molecules which are in combination with nucleic acid may be present in tuberculin solutions. These all have higher mobilities at pH 7.7 and $\mu = 0.1$ in phosphate buffer: -7 to -8×10^{-5} or more, depending on the amount of nucleic acid in combination. They have been called C proteins. It is not yet clear whether the nucleic acid combines only with the native A or B protein, or with the denatured forms A' and B'. It is, however, highly probable that combination does occur with the B and B' types of proteins, since they are the ones which give evidence of the presence of extra imidazole groups and it has been demonstrated (33) that the combination between nucleic acid and at least some of the protein is through this grouping.

A fraction, TPT-18E(A), containing 5.9 per cent nucleic acid, was also a single homogeneous electrochemical component (see first fraction in figure 3) but was reported by Mrs. Bevilacqua to be very inhomogeneous in sedimentation and diffusion (see C protein in table 1). Its pH-mobility curve (C in figure 1) shows that if the enhanced mobility was due to combined nucleic acid, this nucleic acid was bound in a sufficiently strong union that it did not separate above pH 5, as had been found with a protein-nucleic acid preparation previously studied (33). It may, therefore, be considered a true nucleoprotein.

B. Denaturation of the protein of tuberculin

It has been pointed out above that denatured forms of the A and B proteins have been identified. They were no longer soluble at all pH values but were precipitated at pH 4 to 5, and the mobility curves indicated a shift in the isoelectric points. Evidence also exists that with this denaturation there is an uncoiling of the molecules and even degradation. Under certain conditions, then, these denatured molecules may combine with molecules like themselves, forming long random chains and fibrous bundles in which the net charge may be the same as that of the original A' and B' molecules, or with each other, in which case the net charge would lie between that of the A' and B' molecules, as is evident in the case of the D molecules (see table 1). Mixtures of all such molecules would explain the broad electrophoretic peaks observed in solutions of heated tuberculin.

This picture of uncoiling of the protein, followed by splitting and then polymerization, fits well with modern concepts of protein denaturation in general. Bull (3) has stated, "All of the results I have reported dealing with denaturation indicate, as nearly as I can judge, that the reaction consists essentially in a rupture of certain linkages in the compact spherical native protein molecule with the production of a very asymmetric polar molecule. The degree of extension of this molecule is probably to a certain extent dependent on the pH of the solution; at some distance from the isoelectric point the presence of many groups of like electrostatic charge would tend to produce an extended structure, while in the neighborhood of the isoelectric point, the structure would probably be more compact." Astbury, Dickinson, and Bailey (2) showed by means of x-rays that the denaturation of egg albumin and seed globulins proceeded first by disappearance

of the shorter spacing and then further breakdown and aggregation into fibrous bundles. Mirsky and Pauling (24) explained denaturation as primarily a rupture of the labile hydrogen bonds, with a resulting uncoiling of the molecule.

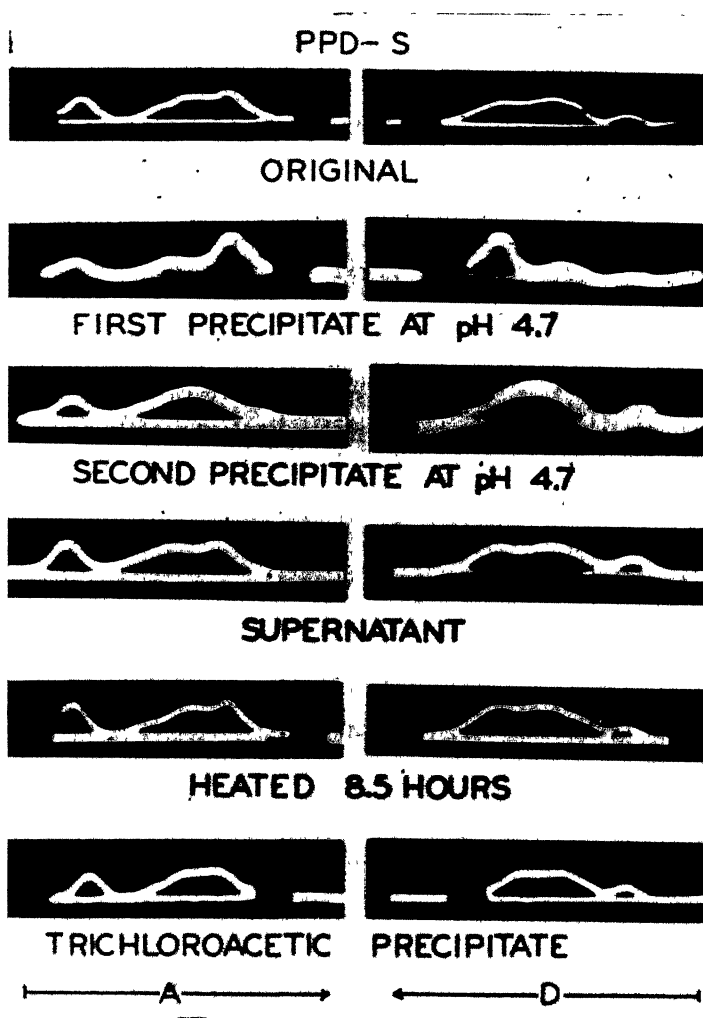


FIG. 2. Electrophoretic diagrams of heated tuberculin protein PPD-S treated in various ways.

It is not yet clear whether the B protein is derived from the A by the unmasking of imidazole groups, or whether it is a naturally occurring protein of different chemical composition. No published evidence for the transformation of one into the other so far exists.

Attempts to cause such a transformation have so far yielded negative results. For example, figure 2 gives first the electrophoretic diagram of PPD-S, a fraction

made from heated tuberculin (36), and shows the presence of two types of protein with mobilities about -3.5×10^{-5} and -5.7×10^{-5} in phosphate buffer at pH 7.7 and $\mu = 0.1$. The fraction which precipitates from this solution at pH 4.7 (second curve) contains mainly a faster fraction. On standing, the supernatant from this precipitation again yielded a small precipitate at pH 4.7, but this contained chiefly a slower component (third curve). The final supernatant contained the two original components, both of which were soluble at all hydrogen-ion concentrations and were probably the native proteins A and B.

If drastic heating were able to transform one protein into the other, one of the components of such heated material (SH, fifth curve) should predominate, but this is not the case. In this experiment PPD-S was evaporated continuously in a boiling water bath for 8.5 hr., with addition of Long's synthetic medium at frequent intervals to maintain the original volume.

Furthermore, precipitation of some of the supernatant with trichloroacetic acid yielded a fraction, shown in the sixth curve, again not very dissimilar from the original material. The detection of small changes, which could only be achieved by quantitative measurements of the areas under the curves was, of course, not advisable in mixtures of proteins so poorly resolved.

While it has so far been difficult to demonstrate a change from the A type to the B type protein in the laboratory, it is the common experience that insoluble or denatured protein readily forms from the soluble type during purification. This fact was clearly demonstrated during the recrystallization of the tuberculin protein (30), and was the reason why crystallization was considered to be an impractical procedure. The factors which lead to the production of the denatured A' and B' type proteins at one time and to the D type of protein, with intermediate mobility, at another time from the same original fraction are not yet clear. For example, during the first fractionation of TPU-84, the fraction which precipitated at pH 4.7 to 5 had a mobility of -6.5×10^{-5} in phosphate buffer at pH 7.7 and $\mu = 0.1$ (see figure 4 and table 4), whereas in a second fractionation carried out in the same way, a fraction with mobility -5.5×10^{-5} resulted.

Further evidence that these fractions, which are insoluble at their isoelectric points, are denatured molecules lies in the fact that they always show a decreased tuberculin potency, as will be discussed in the section on biological reactions.

The apparent great lability of the undenatured tuberculin protein may account for the difficulty so far experienced in attempts to isolate a reasonable quantity of this fraction. In seeking other possible explanations for this difficulty one cannot overlook the additional possibility of association and dissociation among the different proteins of tuberculin as the concentrations vary during the purification processes, in a manner similar to that described for serum proteins by McFarlane (23) and extensively studied by Pedersen (45).

The possibility of denaturation of tuberculin protein as a result of great dilution must be considered because of its great practical importance. Cohn and Edsall (4) warn against high dilution of protein solutions, since the proteins may become denatured and separate into insoluble precipitates or the molecular weight

may be greatly diminished. While chemical data are so far lacking to establish such a denaturation effect in the case of the tuberculin protein, it is highly probable that it may occur. It is recognized, for example, that the high dilutions of tuberculin protein (1 ml. = 0.2 γ) used for the skin test in detecting tuberculosis must be made fresh on the day of test in order to avoid loss in potency.

C. Separation of the protein molecules

It is obvious from the results obtained that those protein molecules which can be precipitated at pH 4 to 5 are the denatured proteins which probably have been formed from native protein. The separation of the native molecules from these molecules, which exist in various stages of denaturation with consequent different chemical properties, is very difficult.

An attempt was made to separate the proteins in a trichloroacetic acid precipitate (TPT-18) of unheated tuberculin protein (38) containing about 6 per cent of nucleic acid by fractionation at various hydrogen-ion concentrations according to solubilities. Figure 3 shows the electrophoretic diagrams, at pH 7.7 and $\mu = 0.1$ in phosphate buffer, of some of the fractions obtained. In all cases the electrophoresis lasted 1 hr. By noting the distance traveled by each component from the δ or ϵ boundary one can see that the sloping heterogeneous curve of the original material has been resolved into its different components. Table 3 gives the mobilities and principal solubilities of the fractions shown in the curves.

Separation of the proteins in an unheated tuberculin may prove to be a more practical problem, since it is obvious from the electrophoretic diagrams (figure 4) that the components can be more satisfactorily resolved than in the case of the denatured fraction. Figure 4 shows that varying proportions of the two types of soluble proteins, A and B, can be found in different preparations. In the case of the TPU-84 unheated tuberculin, when the fraction insoluble at pH 4.7 (first curve in figure 4) was removed, there remained two distinct soluble proteins (second curve). When this soluble supernatant was precipitated by one-fourth saturation with ammonium sulfate, the two types of proteins, A and B, precipitated in equivalent amounts (third curve), whereas half saturation with ammonium sulfate gave a product whose picture was similar to the original. Attempts are now being made to separate these two proteins. The last two curves show fractions in which the A protein predominated. All of the curves in figure 4 represent electrophoresis for 1 hr. in phosphate buffer at pH 7.7 and $\mu = 0.1$; the actual mobilities are recorded in table 4.

D. Biological reactions of the protein of tuberculin

Tuberculin protein is highly potent in causing a local skin reaction in human beings who have been infected with the tubercle bacillus. For this reason it has been very useful as a diagnostic agent for helping in the detection of tuberculosis. As a result of infection with the organism, an animal or human being develops a hypersensitivity to the protein fraction. The hypersensitivity developed to the protein is often of high degree and may persist throughout life.

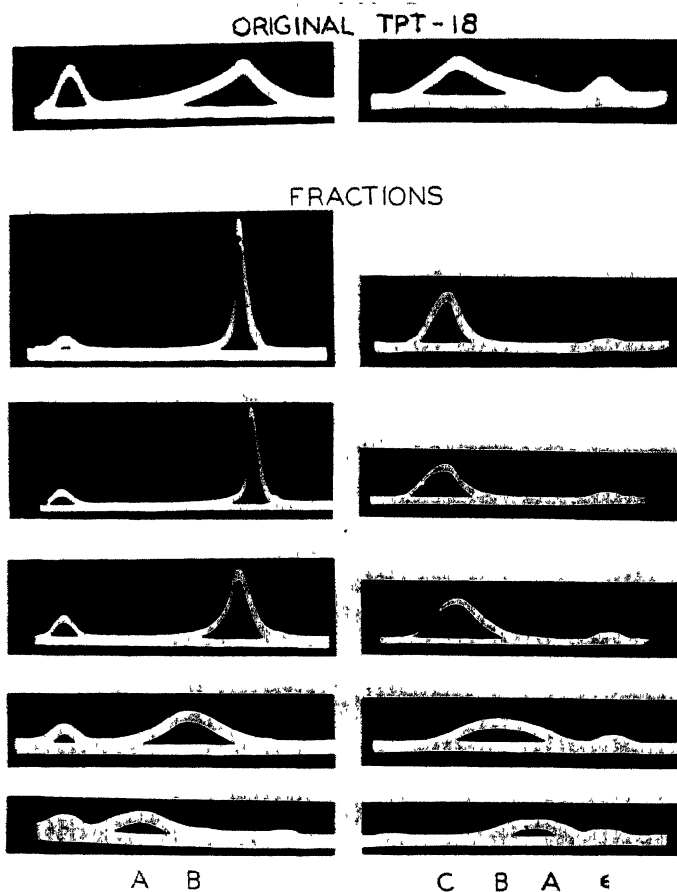


Fig. 3. Electrophoretic diagrams of tuberculin protein, TPT-18, and fractions thereof

TABLE 3

Mobilities of fractions separated from a trichloroacetic acid precipitate

FRACTION	SOLUBLE AT pH	INSOLUBLE AT pH	MOBILITIES $\mu \times 10^5$
Original (TPT-18).....			-8.0 (chief component)
E(A)*.....	11.0	5.8	-8.3
E(less soluble).....	11.0		-8.1
D(C).....	7.6	5.8	-7.6
C1 + D1.....	4.3	Not at all	-6.4
C1.....	2.8	Not at all	-5.2

* As noted earlier in the text, this fraction was very heterogeneous on sedimentation and diffusion.

A protein fraction which has been separated from tuberculin practically free of polysaccharide and nucleic acid has a potency such that 0.02 γ given intracu-

TUBERCULIN PROTEINS

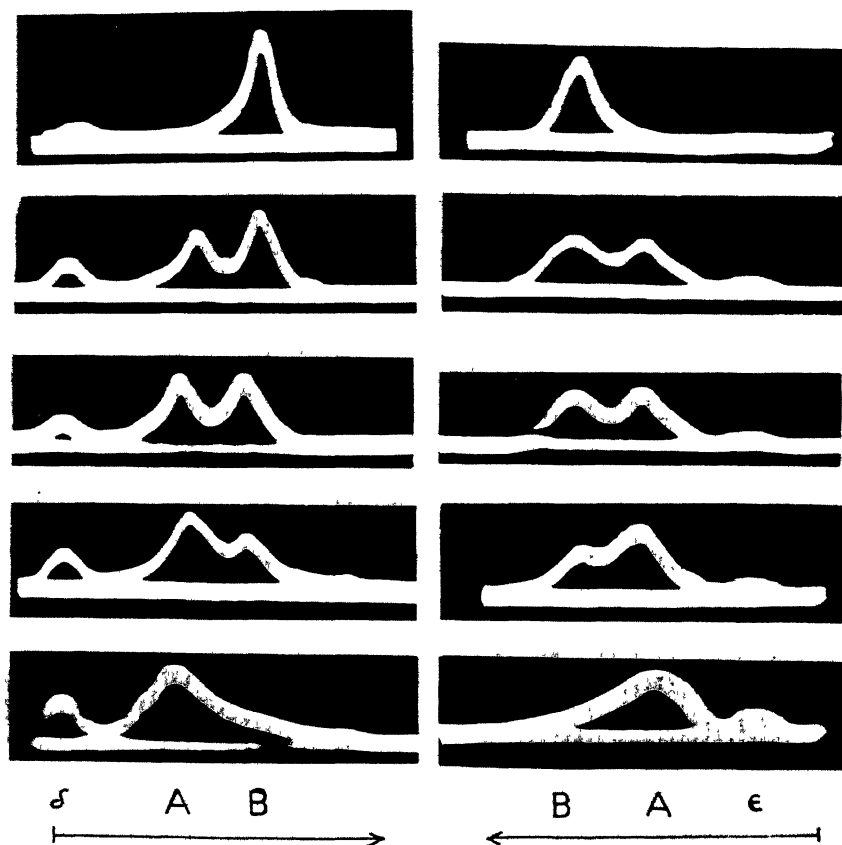


FIG. 4. Electrophoretic diagrams showing varying proportions of proteins A and B in unheated tuberculin fractions.

TABLE 4
Mobilities of fractions separated from unheated tuberculins

FRACTION	MOBILITIES $\mu \times 10^5$	
TPU-84: Precipitate at pH 4.7.	-6.5	
TPU-84: Supernatant	-6.7	-3.9
TPU-84: Precipitate after one-fourth saturation with ammonium sulfate	-6.6	-3.8
M9b: Precipitate after half saturation with ammonium sulfate	-6.3	-4.1
#85C3: Precipitate after half saturation with ammonium sulfate		-3.5

taneously in a volume of 0.1 ml. will give after 24 to 48 hr. a definite area of swelling in an infected person. This dose accordingly has been chosen as a standard first test dose (35, 36). In case no reaction occurs a second test with 5 γ is given, in order to detect a lower degree of sensitivity. No reaction whatever occurs if there has been no infection.

Guinea pigs are less sensitive and require 0.5 to 5 γ to cause an appreciable local reaction. When given intraperitoneally 1 to 1.5 mg. is lethal in 24 to 48 hr. for tuberculous guinea pigs but as much as 100 to 150 mg. is required to kill a normal guinea pig.

Thus it seems that the reaction is due primarily to sensitization but that there may also be a certain degree of primary toxicity involved when large doses are concerned. The suggestion has been repeatedly made (8, 37) that the toxic factor, causing death in tuberculous guinea pigs, may differ from the factor responsible for giving the local skin reaction.

That tuberculin protein is an excellent antigen is obvious from this high degree of sensitization in infected individuals and has also been demonstrated by the fact that it readily stimulates the production of antibodies such as precipitins (31) and causes anaphylaxis (16), uterine strip contraction and bronchial spasm (16), and the Arthus reaction (32). Some tuberculin protein fractions, however, appeared to be less or not at all antigenic (32) but still capable of producing the skin reaction, and these were chiefly isolated from heated preparations.

A careful study (40) of the type of protein that was present in the various preparations which varied in antigenicity and potency revealed the fact that the more native protein, and therefore the larger one, of molecular weight about 32,000, was the most antigenic and gave an early type of reaction reaching its maximum at 24 hr. and fading at 48 hr., whereas the smaller one, of molecular weight about 16,000, was non-antigenic and gave a delayed type of reaction, with maximum size at 48 hr. and less at 24 hr. More recently (39) these two proteins have been identified as the slow and the fast type of protein in electrophoresis. They would be classified, respectively, as the A and the B' proteins described in this paper. Their relationship to the A' and the B proteins remains to be ascertained.

It has been shown (39) that a certain amount of immunological specificity can be demonstrated between the slow and the fast proteins. Fractions which predominated in the slow one caused, when injected repeatedly into rabbits, a definite rise in the γ -globulin of the serum, and this γ -globulin on isolation was shown to contain specific antibodies. Fractions predominating in the fast protein caused a rise in the α -globulin fraction of serum. In these latter experiments the data showed (39) that the removal of a specific precipitate by means of the antigen caused a decrease in the proportion of α -globulin content of the remaining serum in some cases and of albumin in others. With the recent developments reported by Longworth (19), i.e., better resolution of serum components in the longer electrophoresis cell and the separation from albumin of a previously unrecognized component, designated as α_1 , in diethylbarbiturate buffer, it becomes probable that the antibody produced by injection of the fast type of tuberculin protein (B) may really be in an α_1 -fraction rather than part of the albumin.

For similar reasons the antibody produced by injection of the slow type of tuberculin protein (A) may prove on better resolution to exist in a component with mobility between the β - and γ -components rather than in the γ -globulin, as stated.

At any rate, it is obvious that different antibodies are formed to the two types of tuberculin protein. This is a reasonable result when consideration is given to the fact that the two protein antigens (A and B) differ in their polar groups, as indicated by their pH-mobility curves (figure 1).

The significance of these differences for the skin reaction remains to be observed, and this observation awaits the isolation of the pure proteins in adequate quantity for chemical and biological tests on the same fractions. That this type of study may prove of value is suggested by the fact that some confusion already exists in regard to the skin reaction with the present purified fractions. For example, different protein fractions have been shown to have different quantitative potencies. The one, PPD (39), now used as the standard has twice or four times the potency of some previously isolated fractions. It also contains more of the A type of protein. In addition to being more potent per milligram, it also elicits reactions in more people than do other preparations (13, 22). It has been questioned by Furcolow, Hewell, Nelson, and Palmer (10) whether some of these reactions to the larger dose might not be non-specific. In addition to giving larger and more reactions this fraction also gives rise to earlier reactions, which appear as early as 6 hr., as shown by McCarter and Watson (22).

All of these facts argue for the greater antigenicity of the preparation, which has actually been demonstrated (22, 36) by the facts that it causes the production of antibody in animals and, moreover, is shown by electrophoresis to contain a moderate amount of both types of proteins, A and B. It is logical to think that sensitization caused in the infected animal has been by means of the native forms of the protein or proteins and that greater specificity in detecting this hypersensitivity might be obtained through the use of the least denatured proteins, providing this can be done without actually sensitizing the host by means of the test dose. It is obviously important to obtain each type of protein in its native as well as denatured form for the purpose of answering these questions.

Preparations shown to contain considerable amounts of denatured tuberculin proteins have invariably proved to be less potent. For example, the fractions TPT-18E(A) and Q(E), described earlier, when tested intracutaneously in sensitized human beings, gave very much smaller local reactions than did the standard tuberculin protein, even when four times the standard dose was used (see table 5). It was significant that this difference was less marked and even reversed when the larger (5 γ) dose was used.

IV. SUMMARY

Tuberculin contains chiefly three colloidal constituents, protein, polysaccharide and nucleic acid, of which the protein is the most important because of its specific biological reactions in tuberculous animals and human beings.

The polysaccharide and nucleic acid have both been isolated in pure form and their physicochemical properties are described. Neither one gave any signifi-

cant biological reactions, except a specific precipitin reaction between the polysaccharide and sera from animals immunized with the tubercle bacillus.

The protein portion of tuberculin is very complex. Two soluble native proteins (A and B) have been identified, which can be distinguished by their different mobilities in electrophoresis, and which have been shown to contain different amounts of polar groups dissociating between pH 5 and 6, suggesting the presence of imidazole groups. Denatured forms of these two proteins (A' and B') have also been identified. They were insoluble at their isoelectric points and differed from the native proteins in their electrochemical properties. One of these proteins was found to have a molecular weight of 16,000, half that of one of the native proteins, and also to be more elongated in its physical structure.

TABLE 5
Tuberculin potency of denatured tuberculin proteins

SUBSTANCE	DOSE	NUMBER TESTED	NUMBER POSITIVE	NUMBER MISSED	AVERAGE SIZE REACTION
	γ				mm.
Standard.....	0.02	69	56	1	29 x 24 x 2.4
TPT-18E(A).....	0.08	69	44	12	17 x 15 x 1.9
Standard.....	5.00	17	14	0	17 x 14 x 1.8
TPT-18E(A).....	20.00	22	15	4	19 x 17 x 2.0
Standard.....	0.02	57	41	0	34 x 28 x 2.7
Q(E).....	0.08	57	39	2	15 x 14 x 1.7
Standard.....	5.00	16	14	0	20 x 17 x 2.0
Q(E).....	20.00	16	14	0	17 x 15 x 1.9

Other more severely denatured proteins (D), which show extensive elongation and even great polydispersity in the ultracentrifuge, have also been found, chiefly in heated tuberculins. They frequently showed electrochemical homogeneity, with mobility intermediate between the mobilities of the two types of native protein.

Nucleoproteins with mobility much higher than that of the protein have also been studied.

Tuberculin potency can be ascribed to the native proteins and to those fractions in the early stages of denaturation. Antigenicity is a property belonging chiefly to the native forms. Some immunological specificity has been noted for the two different main types of protein. The more denatured the proteins are chemically, the lower is their potency for eliciting the tuberculin skin reaction.

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DETERMINATION OF OXYGEN IN ORGANIC COMPOUNDS¹

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I. INTRODUCTION

Although oxygen is one of the most commonly occurring constituents of organic compounds, and although methods for its direct determination have been the subject of extensive investigation, this element is still most frequently determined by difference. It is apparent that the methods available for its direct determination are not wholly satisfactory, else they would be more widely applied in preference to a determination by difference. The determination by difference places the sum of all the errors upon oxygen, and eliminates the possibility of checking the analysis by totaling the determined constituents. A reliable direct determination would permit such a check, and would be particularly valuable for the purpose of differentiating between compounds which have similar carbon and hydrogen content, but which differ in their oxygen content.

The determination of oxygen by difference becomes more unsatisfactory with increasing complexity of the substances being analyzed. The greater the number of constituents to be determined, the more desirable is a direct method for oxygen which will serve as a check on the analysis. In recent years, therefore, attention has been directed to the problem of determining oxygen directly in

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organic compounds. There often are cases where a determination of the oxygen content would be a more positive index to the purity or nature of the substance under consideration than the usual elemental determinations. The need for a direct determination of oxygen in coal, for example, has been pointed out frequently (11, 19, 33, 60, 97).

The fuel technologist is vitally interested in the direct determination of oxygen in coal; the knowledge of the exact oxygen content would aid him in the study of coal formation and utilization, and would permit the calculation of more precise heat balances. The indirect determination of oxygen in coal is complicated by the presence of inorganic oxides and water of composition, as well as by the number of elements which must be determined before the amount of oxygen can be calculated by difference. The result is usually regarded as a poor approximation. It is significant that many of the papers dealing with the direct determination of oxygen in organic compounds have come from laboratories devoted to coal research. A method for the direct determination of small amounts of oxygen would be of value in studying the nature and oxidation of such materials as lubricating oils, asphalts, and rubber.

To present the other side of the picture, Dennstedt, forty-five years ago, in his account of the development of organic analysis (15), had the following to say of a method for the determination of oxygen in organic compounds: "That one, however, can manage very well without such a method is seen in the history of the development of organic chemistry. One sees no place where the progressive development could have been checked by the lack of such a method."

However, the almost one hundred papers published on the determination of oxygen during the past two decades have indicated the need for such a method. Despite this amount of work, no entirely satisfactory method has yet been developed. This fact is succinctly stated by Hans Meyer (59) in the latest (1938) edition of his well-known book on organic analysis: "The methods developed to the present for the determination of oxygen are extremely bothersome and not of general applicability, so that they are scarcely ever used by anyone besides their discoverers."

It is the purpose of this paper to present a critical examination of the available methods for the direct determination of oxygen on the basis of (1) the experience of those who have used them and (2) the inherent simplicity or complexity of the technic and the precision and accuracy possible. The methods which are described in the literature may be conveniently classified under three main headings on the basis of the method of decomposition, and they are so discussed in this paper. The most important of the technics suggested have been those based upon complete oxidation of the compound with a measurement of the oxygen consumed, catalytic hydrogenation to form water, and thermal decomposition over carbon to form carbon monoxide. It is the hope of the authors that this review may aid in the further development of successful methods for solving the problem.

Previous reviews (1, 7, 14, 15, 59, 95) of the work on the determination of oxygen in organic compounds have been fragmentary and uncritical; the most helpful

has been the short introduction to a paper on the topic by Kirner (30). The review by Skanovi-Grigor'eva (86) was, unfortunately, not available to the authors; the length of the review suggests that it might be valuable, although the abstract describes it as chiefly a review of German literature. The present review is based in part on a previous paper by the authors, only an abstract of which was published (20).

In the discussion which follows, deviations of analytical results from the calculated values are expressed in terms of absolute percentage, unless otherwise stated.

II. METHODS BASED ON COMPLETE OXIDATION OF THE COMPOUND

These methods depend upon completely oxidizing the sample by means of an inorganic oxidizing agent or gaseous oxygen. The amounts of water and carbon dioxide formed in the oxidation are determined, giving, by calculation, the total amount of oxygen in the products of combustion. From this value is subtracted the quantity of oxygen supplied by the oxidizing agent, the difference being the amount of oxygen which was present in the substance being analyzed.

A. OXIDATION WITH AN INORGANIC OXIDIZING AGENT

1. *Dry oxidation*

The earlier methods for the determination of oxygen in organic compounds were based upon oxidizing the organic substance with materials such as potassium chlorate, cupric oxide, silver iodate, potassium dichromate, or other agents which readily yield oxygen. One such method was proposed as early as 1811 by Gay-Lussac and Thenard (23), who oxidized organic compounds containing carbon, hydrogen, oxygen, and nitrogen with a known amount of potassium chlorate. From a determination of the products of combustion and the amount of potassium chlorate unconsumed, the amount of oxygen in the compounds could be calculated. Carbon and hydrogen were determined at the same time.

Persoz (69) oxidized the sample in a sealed tube with mercuric sulfate and collected the resulting gases in a bell jar. The carbon dioxide, sulfur dioxide, nitrogen, water, and mercury formed in the reaction were determined. From these data the carbon, oxygen, nitrogen, and sulfur present in the sample were calculated.

Baumhauer (3) developed a method for determining oxygen in compounds containing carbon, hydrogen, oxygen, and nitrogen, using cupric oxide to oxidize the compound. The amount of oxygen supplied by the cupric oxide was determined by heating the residual oxidizing agent in oxygen and measuring the contraction in the gas volume. From the determination of the water and carbon dioxide formed and the oxygen removed from the cupric oxide during combustion, the amount of oxygen in the compound was calculated. In a modification of the method (4), the reduced copper was reoxidized with a known weight of silver iodate. The excess oxygen from the silver iodate was absorbed upon a mat of copper and determined by reducing the resulting copper oxide in a stream of

hydrogen and weighing the water formed. With the addition of an azotometer, carbon, hydrogen, oxygen, and nitrogen could be determined on the same sample.

Boswell (9) also utilized combustion with cupric oxide in a stream of nitrogen, but greatly simplified the determination of the oxygen removed from the cupric oxide. The sample was vaporized in a stream of nitrogen and passed over a known amount of cupric oxide suspended on asbestos. The oxidizing power of the cupric oxide was previously determined by reduction with hydrogen. The products of combustion were absorbed and weighed, and the loss in oxidizing power of the cupric oxide was determined by reduction with hydrogen as before. Simple calculations gave the amount of oxygen in the substance analyzed. Hydrogen was determined simultaneously. The results were accurate to within about 0.3 per cent for hydrogen, and to within 0.3 to 1.0 per cent for oxygen.

Stromeyer (91) determined oxygen in compounds containing carbon, hydrogen, oxygen, nitrogen, sulfur, and chlorine, using a mixture of cupric oxide and sodium carbonate as oxidizing agent. After oxidation, the contents of the tube were dissolved in hydrochloric acid and ferric sulfate solution was added. The ferrous ion formed, as determined by titration with potassium permanganate solution, was a measure of the amount of oxygen given up by the cupric oxide. The results were always low and the method failed entirely with nitrates and nitro compounds.

Still another method for determining the amount of oxygen given up by cupric oxide was suggested by Prout (72). The used cupric oxide was agitated with dilute sulfuric acid and an amount of copper equivalent to the oxygen given up remained undissolved, because cuprous oxide when treated with dilute sulfuric acid yields cupric sulfate and metallic copper, the latter being insoluble in cold dilute sulfuric acid. A similar technic was employed by Porret (71).

A recent paper by Trautmann (92) describes the complete analysis of urea in a single operation, the oxidation being carried out with cupric oxide powder in a measured volume of nitrogen. Air is passed over heated copper in a glass tube; the resulting nitrogen is dried by passage over phosphorus pentoxide and is then collected in a gas buret. This nitrogen is used to sweep the combustion tube and train during oxidation of the sample with cupric oxide, and is collected in a second gas buret together with nitrogen from the urea. The increase in volume represents the urea nitrogen. The weights of water and of carbon dioxide formed in the combustion are determined by absorption with phosphorus pentoxide and soda lime. From these data, together with a knowledge of the oxygen supplied by the cupric oxide, the complete elementary analysis of the urea is obtained.

A mixture of litharge and calcium phosphate was employed by Maumene (45) as the agent for completely oxidizing organic compounds. The sample was fused with litharge and a small amount of calcium phosphate. The litharge furnished the oxygen necessary to complete the oxidation of the compound to water and carbon dioxide, which were determined by the usual absorption methods. A determination of the amount of metallic lead formed gave the amount of oxygen necessary to complete the oxidation. The amount of oxygen in the compound being analyzed could then be calculated. No data were given

concerning the types of compounds analyzed, nor were results reported to indicate the precision and accuracy possible.

Mitscherlich (63, 64, 65) developed an ingenious although exceedingly complicated technic, comprising combustion with mercuric oxide. The water, carbon dioxide, and, in the case of nitrogen-containing compounds, nitric oxide were absorbed and weighed. The resulting metallic mercury was sublimed into a small tube and weighed, thus supplying information as to the amount of oxygen required to complete the combustion. It was claimed that sulfur, phosphorus, chlorine, bromine, and iodine, as well as carbon, hydrogen, oxygen, and nitrogen, could all be determined by a single combustion. The former elements were retained by the mercury and were determined in the residue.

2. Wet oxidation

A number of wet oxidation methods have been proposed. Ladenburg (40) used concentrated sulfuric acid and silver iodate in a sealed tube at elevated temperatures, determining the excess silver iodate by an iodide-thiosulfate procedure. Good results were obtained with compounds of carbon, hydrogen, and oxygen. Phelps (70) employed a digestion mixture of chromic acid and sulfuric acid for the oxidation, using a known weight of potassium dichromate. After the oxidation was complete, the excess dichromate was determined by adding hydrochloric acid, absorbing the liberated chlorine in an arsenite solution, and determining the excess arsenite with a standard iodine solution. Compounds of carbon, hydrogen, oxygen, and nitrogen were studied, the poorest results being obtained with the nitrogen-containing compounds. The method was not applicable to volatile or difficultly oxidizable compounds.

Strebinger (90) determined oxygen by finding the amount of potassium iodate required to complete the oxidation of the substance. An independent determination of the other constituents was necessary. The sample was heated with concentrated sulfuric acid and a weighed amount of potassium iodate until no further evolution of iodine vapor occurred. The excess potassium iodate was then determined by titration with sodium thiosulfate solution. Good results were reported for compounds containing not only carbon, hydrogen, and oxygen, but also halogens and sulfur. Certain nitrogen-containing compounds gave poor results: namely, compounds which gave poor results in the Kjeldahl determination, such as nitro, azo, and heterocyclic compounds, hydrazones, and osazones. Stanek and Nemes (87, 88), who adapted Strebinger's technic to a micro method, experienced the same difficulty.

Although a great deal of research has been done upon both wet and dry oxidation methods with a variety of oxidizing agents, none of these methods has been found to be very satisfactory. In addition to disadvantages peculiar to each of the methods described, there are certain general objections to this technic. A number of weighings are required, usually accompanied by excessive calculation, and the determination of the oxygen consumed is frequently very indirect. The assumption is made that all of the oxygen is converted to carbon dioxide, whereas it is possible that a portion is converted to carbon monoxide, unless provision is

made to insure oxidation of the latter. The method is usually inapplicable to nitrogen-containing compounds, owing to the formation of various nitrogen oxides and other compounds. It is necessary that the oxidizing agent used be free from moisture and impurities. This condition is often difficult to fulfill, resulting in inaccuracy in determining the oxidizing power of the reagent.

The advantages of the method are simplicity of apparatus and manipulation, especially with the wet methods, and the possibility of determining carbon, hydrogen, and oxygen simultaneously. The latter, however, might be considered a disadvantage in some cases, since it is not only a possibility but a necessity that carbon and hydrogen also be determined. That is, the method does not permit a determination of oxygen only, although the analyst might frequently be interested in no constituent other than oxygen.

In view of the many disadvantages of the method compared to the few advantages, and particularly the many probable sources of error under all except ideal conditions, it is not surprising that little attention is now given to this technic.

B. COMBUSTION WITH A KNOWN VOLUME OF OXYGEN

Glockler and Roberts (24) first proposed the determination of oxygen in organic compounds by burning the substance in oxygen, followed by a measurement of the products of combustion and the volume of oxygen consumed. Carbon, hydrogen, and oxygen were thus determined simultaneously. They arranged the combustion train as a closed circulating system to insure complete combustion with the minimum amount of oxygen. Only platinized asbestos was used as filling for the combustion tube. The commonly used cupric oxide was omitted in order to avoid the possibility that the copper resulting from reduction during combustion might be reoxidized to a different extent than formerly. The ordinary carbon-hydrogen assembly was used with the introduction into the closed system of a Ramsay buret, for measurement of the oxygen before and after combustion, and of a Sprengel pump, previously calibrated to produce the usual flow of oxygen through the combustion tube. The oxygen was produced from solid potassium permanganate. The necessary temperature and pressure corrections were made to convert the volume to the weight of oxygen consumed. The oxygen content of the sample was readily calculated from the weight of the products of combustion and the weight of oxygen consumed. A period of 45 min. was allowed for complete combustion and 15 min. in addition to sweep the combustion tube thoroughly, 2 hr. being required for a complete analysis. Samples of approximately 30 to 35 mg. were employed.

Data are given for four determinations on benzoic acid, the average of the four being 0.69 per cent low for oxygen. Perhaps the best evaluation of the method as applied by the authors is their statement that "one would naturally take the per cent of oxygen as obtained by difference as the more accurate figure after having demonstrated by this method that oxygen actually is contained in a given compound."

In the same year, Dolch (16) proposed a variation of the method which depends upon combustion of the sample in an oxygen-nitrogen mixture of known or

determinable composition, e.g., air, and determination of the carbon dioxide content of the effluent gases. Dolch and Will (17) gave further details in a later publication. The composition of the oxygen-nitrogen mixture serves as a reference and a direct measurement of the oxygen consumed is not made; it is not necessary to know the volume of the combustion gases, but only the percentage of carbon dioxide in the gas mixture after combustion, plus the carbon and hydrogen content of the sample. This procedure offers no advantages over that presented by Glockler and Roberts and is subject to greater errors; consequently, the attention of subsequent investigators, except for criticism by Beek and Waard (5) and by Schuster (82), has been confined to applying and improving the technic of Glockler and Roberts.

In the following six years, 1928-1934, a number of papers on the method appeared. Dumke (19) extended the method to the analysis of cane sugar, urea, trinitrobenzene, and coal. It was assumed that the nitrogen in the urea, trinitrobenzene, and coal samples was liberated during combustion as elemental nitrogen, and that the sulfur in the coal other than that remaining in the ash was converted to sulfur trioxide, and was retained as sulfuric acid in the phosphorus pentoxide tube. The average of two determinations on urea was 1.1 per cent high for oxygen. A single determination on trinitrobenzene was 1.3 per cent high. The averages on three samples of coal varied from 0.7 per cent low to 1.5 per cent high for oxygen, compared to a calculation by difference. The percentage of hydrogen was high in almost every determination.

Stock, Lux, and Rayner (89) adapted the method to the determination of small quantities of hydrogen and oxygen in active charcoal. Hellman (26) described a volumetric determination of the combustible constituents of solid and liquid fuels, based upon burning the fuel in a closed system with oxygen, and measuring the contraction in volume and the carbon dioxide produced. Dolch and Will (18) have also reported on the method as applied to the determination of oxygen in coal products.

Kirner (30) has made a critical study of the method, and adapted it to the microdetermination of oxygen. He pointed out that previous investigators neglected to make accurate corrections for the effect of temperature and pressure on the gas volume, and concluded that "the apparent concordance of their results was largely due to fortuitous compensation of errors of considerable magnitude." In analyzing the data presented by Glockler and Roberts (24), Kirner notes that the mean error of their carbon determination was -0.12 per cent, and of their hydrogen determination $+0.41$ per cent, which should result in a high value for oxygen; however, the mean error reported for oxygen was -0.69 per cent. The discrepancy is accounted for by an inaccurate measurement of the oxygen consumed, this error more than compensating for the errors in the carbon-hydrogen determination. The results reported by Dumke (19) and Dolch and Will (18) are subject to the same criticism. Kirner emphasizes the necessity for a very precise carbon-hydrogen determination, since errors in the carbon determination are multiplied by 2.7, and those in the hydrogen determination by 8.0 in calculation of the percentage of oxygen.

Kirner's apparatus (figure 1) is a closed system similar to that used by previous investigators, but is adapted to the analysis of 10-mg. quantities. Commercial tank oxygen is passed through the customary purifying train before being admitted to the system. The Pregl universal filling is used for the combustion tube, except that a 40 per cent palladium-asbestos catalyst is substituted for the copper oxide-lead chromate filling, because blank experiments showed that the latter caused a loss in oxygen. Circulation of the oxygen is maintained by a Sprengel

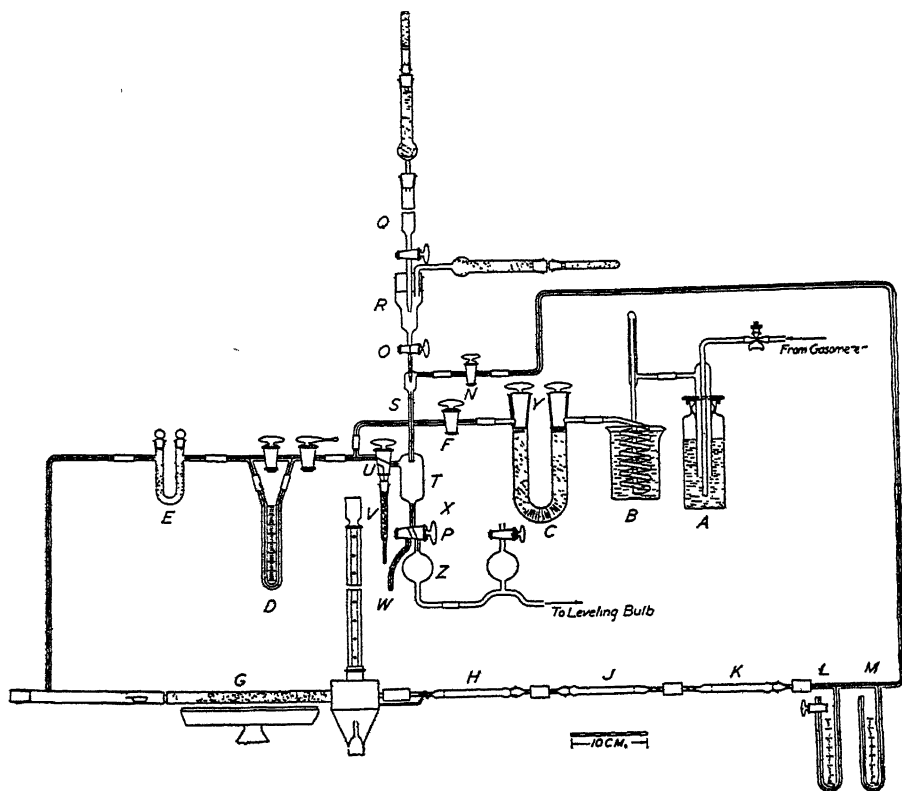


FIG. 1. Apparatus for the determination of oxygen by complete combustion with gaseous oxygen (from Kirner (30)).

pump. Ascarite is used to absorb the carbon dioxide, and phosphorus pentoxide to absorb the water.

The apparatus and technic employed by Kirner are designed to minimize errors in the determination of the oxygen consumed. The volume of the apparatus is measured, and the temperature and pressure are determined at the beginning and the end of each experiment. These precautions were neglected by previous workers. Provision is made for accurate control of the temperature by housing the entire apparatus in a constant-temperature box, with heating elements and fans actuated by a thermoregulator. A temperature constant to

within $\pm 0.02^\circ\text{C}$. may be maintained by this means. Other precautions to give greater precision and accuracy include a flowmeter and manometer in the combustion train to insure the maintenance of the optimum rate of gas flow and pressure, and the running of blanks to determine the volume of oxygen lost by diffusion through the numerous rubber connections (nineteen in all).

Results are given for ten compounds, three of which contained chlorine or bromine. The results for the seven compounds containing only carbon, hydrogen, and oxygen average 0.29 per cent high, and the three halogen compounds average 0.58 per cent high for oxygen. Kirner concludes that a precision of about 0.4 per cent may be obtained in the determination of oxygen, provided that precautions are taken to obtain a determination of hydrogen accurate to within 0.05 per cent and a very accurate carbon determination.

In subsequent publications, Kirner has shown that the method is applicable to the determination of oxygen in compounds containing sulfur (31), in compounds containing nitrogen (32), and in coal (33). Obviously, this method for the determination of oxygen cannot be applied to compounds containing sulfur and nitrogen unless it is known to what extent the sulfur and nitrogen are oxidized. In the case of sulfur compounds, Dolch and Will (18) assumed that sulfur dioxide is the sole product, whereas others (19, 87, 88) have assumed that the sole product is the trioxide. On the basis of available data on the sulfur dioxide-sulfur trioxide equilibrium, it is to be expected that 99 to 100 per cent of the equilibrium mixture would be the trioxide under the conditions existing in the combustion tube. To confirm this, Kirner (31) analyzed samples of diphenyl sulfone and phenyl *p*-toluenesulfonate. The former would be expected to yield sulfur dioxide upon thermal decomposition, provided no further oxidation occurred, and the latter would be expected to give sulfur trioxide. With both compounds, the amount of oxygen consumed was about 99.8 per cent of that calculated, assuming the sulfur to be oxidized to the trioxide, followed by retention of the sulfur trioxide by the silver wire in the combustion tube and by subsequent conversion from sulfite to sulfate by the excess oxygen. Thus it appears reasonably certain that in the analysis of a sulfur-containing compound by this method, sufficient oxygen is consumed by the sulfur to convert substantially all of the latter to sulfate, which is retained by the silver as silver sulfate. Kirner obtained good results employing 0.9978 as a correction factor.

Investigators (19, 22) previous to Kirner who have used the volumetric combustion method to determine the oxygen in nitrogen-containing compounds have assumed that the nitrogen was converted solely to elemental nitrogen. Dumke (19), for example, assumed that the nitro groups in trinitrobenzene were reduced to elemental nitrogen during combustion of the compound by gaseous oxygen. Kirner recognized that this was highly improbable and attempted to determine the fate of the nitrogen under the conditions of the analysis so that the method might be applied to nitrogen-containing compounds. He postulated that the thermal decomposition products of nitrogen-containing compounds might include ammonia, cyanogen, hydrogen cyanide, nitrogen peroxide, nitric oxide, and elemental nitrogen, and that these initial products would yield only nitrogen per-

oxide and nitrogen in proportions which, under given conditions, would be dependent upon the nature of the nitrogen linkage in the substance being analyzed.

To obtain the desired information, five compounds, chosen to give an example of amido, amino, heterocyclic, nitrile, and nitro linkages, were analyzed. It was found that amines and amides gave 26 per cent of the nitrogen as nitrogen peroxide (NO_2) and 74 per cent as elemental nitrogen (N_2), whereas nitriles, nitro, and heterocyclic nitrogen compounds gave 59 per cent as nitrogen peroxide and 41 per cent as nitrogen. The results indicate that, using these empirical ratios, oxygen may be determined in nitrogen-containing compounds with a mean accuracy of about 0.2 to 0.3 per cent.

Kirner applied the information acquired in the analysis of pure compounds to the analysis of coal and coal products (33). Good results were obtained for oxygen in coal when it was assumed that 59 per cent of the nitrogen was converted to nitrogen peroxide, and 41 per cent to elemental nitrogen, suggesting strongly that the greatest part of the nitrogen in coal is in heterocyclic linkage.

The volumetric combustion method for the direct determination of oxygen has many disadvantages. To obtain an accuracy of 0.5 per cent or better, very accurate determinations of hydrogen, carbon, and the oxygen consumed are required. Any error in the determination of carbon or hydrogen is increased several fold. The oxygen consumed must be obtained as the difference between two comparatively large volumes. Such accurate determinations are possible only with complicated and expensive apparatus. If sulfur or nitrogen is present, a separate determination of these constituents must be made. In the case of nitrogen, certain empirical assumptions, which necessitate a knowledge of types of nitrogen linkage in the compound, must be made with regard to the ultimate fate of the nitrogen in the substance analyzed.

The chief advantage of the method lies in the fact that in the hands of a careful and experienced worker, carbon, hydrogen, and oxygen may be determined simultaneously with reasonable accuracy, using apparatus and technic similar to that employed in the Pregl carbon-hydrogen determination. Halogens, sulfur, and nitrogen offer no complications, such as poisoning of the catalyst, except that separate determinations of the latter two elements must be made.

III. DESTRUCTIVE CHLORINATION

Mitscherlich (61) presented a method for the direct determination of carbon, hydrogen, and oxygen by destructive chlorination at red heat, the products being carbon dioxide, carbon monoxide, and hydrogen chloride. The oxygen-containing compounds, carbon dioxide and carbon monoxide, were absorbed in potassium hydroxide solution and cuprous chloride solution. Determination of the hydrogen chloride gave the hydrogen content. Mitscherlich claimed to have made 319 determinations by this method. A modification (62) involved decomposition over potassium hexachloroplatinate in an atmosphere of nitrogen, the products being water, carbon dioxide, hydrogen chloride, and chlorine. A determination of the first three of the products gave the amounts of carbon, hydrogen,

and oxygen present. The procedure was time-consuming and not very accurate. Apparently, no one other than Mitscherlich has ever used the method.

IV. REDUCTION METHODS

A variety of methods has been applied for determining oxygen in organic compounds by reduction; these are best described under several subdivisions which follow. In general, it may be said that reduction is a more direct attack upon the problem than oxidation, because in the reduction methods the products of the reaction contain only the oxygen which was in the compound, rather than the oxygen of the compound plus the amount added to complete the oxidation.

A. REDUCTION BY MEANS OF A METAL

Cretier (13) proposed a method for the simultaneous determination of carbon, hydrogen, and oxygen which involved heating the sample with a known quantity of magnesium to red heat. Oxygen in the sample is retained as magnesium oxide. The excess magnesium is determined by measurement of the volume of hydrogen evolved when the residue is treated with acid, from which data the amount of oxygen in the residue may be calculated. The evolved gases contain hydrogen, methane, and carbon oxides which have escaped reduction, and therefore an analysis of this gas mixture is necessary.

Determinations were reported for only sucrose and acetic acid. The results obtained were very inaccurate, because of the uncertainty of the composition of the resulting residue, and because of the usual errors encountered in gas analysis. The method is not applicable to compounds containing elements other than carbon, hydrogen, and oxygen.

B. HYDROGENATION

1. *The ter Meulen method*

The first mention of the determination of oxygen in organic compounds by hydrogenation appears in a paragraph announcement by Wanklyn and Frank (96) in 1863. However, no details or data were given and the hydrogenation method is commonly known as the ter Meulen method, since ter Meulen was the first to give experimental details and the results of the analysis of compounds (46). The method consists of (1) vaporizing the sample in a stream of hydrogen; (2) cracking or pyrolyzing the compound at a high temperature, yielding products which depend upon the composition of the compound; and (3) hydrogenating these products in the presence of a nickel catalyst between 300 and 400°C. to convert all of the oxygen to water, which may be absorbed and weighed.

The essential parts of the experimental arrangement are the cracking surface and the hydrogenation catalyst; the latter is also referred to as the methanation catalyst or as the reduction catalyst. Figure 2, which will be discussed later, illustrates a typical apparatus used in the catalytic hydrogenation method for the determination of oxygen in organic compounds. The presence of elements other than carbon, hydrogen, and oxygen in the sample necessitates means for

removing the hydrogenation products of these elements or for correcting for their effect on the oxygen determination. Consideration of this can be deferred until later.

Since the publication of ter Meulen's original paper in 1922, the hydrogenation method has been studied extensively by ter Meulen and others. Many improvements have been made, including the extension of the method to compounds containing nitrogen, sulfur, and halogens. Of the almost fifty papers which have been published on this method, some are polemical, deriding or praising the method; one interesting series consisting of three such communications (5, 51, 98) is the result of an article by Dolch and Will (17) in 1931 criticizing the original ter Meulen method adversely. Many authors have discussed the method in connection with the analysis of coal and its products (2, 5, 6, 10, 11, 17, 29, 51, 68, 81, 82, 83, 95). The majority of the papers, however, have dealt with the best cracking and hydrogenation or methanation catalysts for the process and the optimum conditions for carrying out the cracking and hydrogenation reactions.

The reactions involved in the method are essentially very simple. The sample is pyrolyzed in the vapor phase or on the cracking surface to carbon monoxide, carbon dioxide, water, methane and other hydrocarbons, free carbon, and solid organic residues. The presence in the sample of elements other than carbon, hydrogen, and oxygen results in the formation of other products. The oxygen is supposedly present in the pyrolysis products only as water and carbon oxides. On passage of these products over the hydrogenation catalyst, the oxygen in the carbon oxides is converted to water according to the reactions:

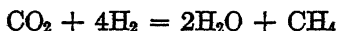
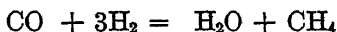


Table 1 lists some of the more important modifications of the ter Meulen method that have been proposed, but this list is not exhaustive. The nature and temperature of the cracking surface and of the hydrogenation catalyst proposed, used, or discussed are given, together with elements which may be present when using the technic described.

In his earliest descriptions (46, 47) of the method, ter Meulen employed nickel suspended on asbestos, maintained at 350°C., as the only catalyst. Samples of 100 mg. were used. The oxygen of the compound was converted to water and carbon dioxide, which were absorbed and weighed; the results on four compounds were accurate to ± 0.5 per cent. In subsequent publications ter Meulen introduced the cracking catalyst, using platinized asbestos (48) or plain asbestos (49). The use of more active forms of reduced nickel improved the final conversion to water; the inconvenience of freeing the asbestos used as support for the nickel from its moisture was avoided by the use of a nickel boat filled with reduced nickel (58). The publication of a monograph by ter Meulen and Heslinga (54) on catalytic methods for the combustion analysis of organic compounds and its immediate translation from the original Dutch into French and German (55, 56)

TABLE 1
Determination of oxygen by catalytic hydrogenation

DATE	REFERENCE	SAMPLE* SIZE	CRACKING SURFACE AND TEMPERATURE	HYDROGENATION CATALYST AND TEMPERATURE	ELEMENTS PRESENT
1863	Wanklyn, Frank (96)				C, H, O
1922	ter Meulen (46, 47)	b		Nickel-asbestos, 350°C.	C, H, O, N, S, X
1924	ter Meulen (48)	b, c	Platinum-asbestos, 1000°C.	Nickel-asbestos, 350°C.	C, H, O, N, S
1926-7	ter Meulen (54, 55, 56, 57)	c	Asbestos, ca. 1000°C.	Nickel and asbestos, 350°C.	C, H, O, N, S, X
1930	ter Meulen <i>et al.</i> (58)	c	Platinum-asbestos, 1000°C.	Powdered nickel in nickel boat, 350°C.	C, H, O, N, S, X
1930	Schuster (81)		Platinum-asbestos, 1000°C.	Nickel, 350°C.	Applied to coal
1931	ter Meulen (50)		Asbestos, red heat	Powdered nickel in nickel boat, 350°C.	C, H, O, N, S, X
1933	Russell, Fulkon (73)	c	Platinum-quartz, 750°C.	Thoria-nickel, 350°C.	C, H, O
1934	ter Meulen (52)	c		Thoria-nickel, 350°C.	C, H, O, S, Cl
1934	Lacourt (36)	c		Thoria-nickel, 400°C.	C, H, O, N, S
1934	Russell, Marks (74)	c		Thoria-nickel, 350°C.	C, H, O, N
1935	Gauthier (22)		Platinum-quartz, 750°C.	Nickel-pumice	C, H, O
1935	Marks (44)	b, c	Pumice, nickel spiral, 650°C.	Thoria-nickel, 450°C.	Oils
1936	Hennig (27)	a	Platinum-quartz, 750°C.	Nickel-pumice	C, H, O
1936	Inaba, Abe (29)	a	Platinum-silica gel, 900-950°C.	Thoria-nickel, 300-350°C.	Applied to coal
1936	Lacourt (37)	a	Nickel spiral, red heat	Thoria-nickel, 350-400°C.	C, H, O, S
1936	Russell, Marks (75)	c	Platinum-quartz, 750°C.	Thoria-nickel, 350°C.	C, H, O, N, S
1937	Afnas'ev (2)	c	Platinum-quartz, red heat	Thoria-nickel, 350°C.	Coal; C, H, O, etc.
1937	Goodloe, Frazer (25)	b, c	Nickel chromite, 750°C.	Nickel chromite, 400°C.	C, H, O, N, S, X
1937	Kirner (34)	a	Nickel gauze roll, 1000°C.	Thoria-nickel, 350°C.	C, H, O
1937	Lacourt (38)	a		Thoria-nickel	C, H, O
1937	Lacourt (39)	a	Nickel-chromite, red heat	Nickel chromite, 450°C.	C, H, O, N, S, X
1937	Lindner, Wirt (42)	a	Nickel spiral, 950°C.	Nickel, 350°C.	C, H, O, N, S, X
1937	Unterzaucher, Bürger (94)	a	Platinum-quartz, 1000°C.	Thoria-nickel, 300°C.	C, H, O
1938	Unterzaucher, Bürger (95)	a	Platinum-quartz, 1050°C., and nickel spiral, 650°C.	Thoria-nickel, 300°C.	C, H, O, N, S, coal
1940	Bürger (12)	a	Quartz wool, 1100°C.	Thoria-nickel, 300°C.	C, H, O, N, S, X
1941	Morikawa <i>et al.</i> (66, 67, 68)	c	Platinum-silica gel, 950°C.	Thoria-nickel, 350°C.	C, H, O, N, S, coal
1941	Shriner (84)	b, c	Nickel chromite, 750°C.	Nickel chromite, 400°C.	

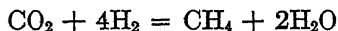
* Sample size: a = 10 mg. or less; b = 10 mg. to 0.10 g.; c = 0.10 g. or over.

served to bring the method for the determination of oxygen to the attention of chemists. A second edition in French (57) soon followed.

Dolch and Will (17) attempted to apply the ter Meulen method to the determination of oxygen in oxalic acid, with very unfavorable results. In four analyses, the experimentally determined values varied from 72.5 to 75.2 per cent compared to a theoretical value of 76.2 per cent oxygen. On the basis of their experimental work and theoretical considerations, Dolch and Will made the following indictment of the method:

(1) The method fails to give accurate results in the analysis of a simple substance even when the directions of ter Meulen are carefully followed.

(2) A large proportion of the oxygen of the substance being analyzed occurs in the hydrogenation products as carbon dioxide rather than as water, whereas ter Meulen claims that the reaction



goes very nearly to completion under the conditions of the analysis.

(3) The hydrogenation products of a coal sample contain an appreciable amount of carbon monoxide, as determined by reaction with iodine pentoxide, for which no provision is made in the ter Meulen method.

(4) ter Meulen fails to give clearly the optimum reduction temperatures for preparation of the catalyst, and the optimum temperature for hydrogenation.

(5) Their conclusions are supported by Schuster (82), who states that the method is unsatisfactory for the determination of oxygen in coal.

To these charges the following reply was made (51):

(1) Literature cited shows the results of forty-nine analyses of thirty-three compounds with a maximum error of 0.1 per cent.

(2) ter Meulen gives the results of a determination of oxygen in oxalic acid as 76.0 per cent compared to the theoretical value of 76.2 per cent. No carbon dioxide was obtained in this experiment. The possibility of error due to too rapid volatilization of the sample, use of too little hydrogen, or an inactive catalyst is mentioned.

(3) It is known that unsaturated hydrocarbons result from the dry distillation of coal and that these products will react with iodine pentoxide, thus accounting for the "carbon monoxide" found by Dolch and Will.

(4) ter Meulen cites the pages of his book on which he gives the proper temperatures for reduction of the catalyst and for carrying out the hydrogenation reaction.

(5) Schuster's objections to the ter Meulen method are solely on the grounds of its inapplicability to coal analysis because of ash reactions, and the reference cited by Dolch and Will actually refutes their claims, since Schuster obtained excellent results for the determination of oxygen in benzoic acid.

Beek and Waard (5) also came to ter Meulen's defense, stating that they obtained results within 0.25 per cent of theory when using the method with samples of 100–200 mg. They hydrogenated a 50-mg. sample of oxalic acid over a period of 1 hr., and obtained no carbon dioxide. Beek and Waard main-

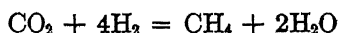
tain that ter Meulen has given the experimental details clearly, and attribute the failure of Dolch and Will to too rapid heating of the sample, against which ter Meulen expressly warned. It was not intended that a large proportion of the oxygen should be weighed as carbon dioxide, as in the work of Dolch and Will. When the amount of carbon dioxide formed is small, no carbon monoxide is obtained.

Beek and Waard also took this opportunity to criticize the method proposed by Dolch and Will for the determination of oxygen (see II B), stating that it could be shown by calculation that the method would not give accurate results.

In a later publication, Will (98) attempted to explain the reason for his and Dolch's failure with the method. He stated that the only respect in which they failed to follow ter Meulen's directions was with regard to the temperature of reduction of the catalyst, since they did not consider ter Meulen's "approximately 350°C." as a precise statement and consequently employed a lower temperature. Will also believed that part of their difficulty was due to too large a sample size. He insisted that the presence of carbon monoxide in the exit gases in their experiments was proven beyond question, and pointed out that the presence of unsaturated hydrocarbons as products of a catalytic hydrogenation, as suggested by ter Meulen, is very unlikely. Will questioned the utility of a method wherein so many factors, and particularly the catalyst, must be subjected to such careful control.

Regardless of whether the criticism leveled by Dolch and Will was justified, it is fortunate that subsequent investigation of the method has resulted in improvements which have largely obviated the difficulties experienced by these investigators.

The technic of determining oxygen in organic compounds by catalytic hydrogenation received a major advancement in the suggestion made by Russell and coworkers (44, 73, 74, 75) that the nickel methanation catalyst prepared by the reduction of nickel oxide be replaced by a promoted nickel catalyst obtained by the reduction of a mixture of nickel and thorium oxides prepared in a special manner. The basis for use of this catalyst is found in a discussion of the promoter action of thoria on nickel catalysts by Russell and Taylor (76), in which the reaction



was studied on promoted and unpromoted, supported and unsupported, nickel catalysts. This application of a study of the nature of catalytic surfaces to the determination of oxygen in organic compounds is an outstanding example of how fundamental theoretical or physical chemical knowledge can be used to improve and develop an analytical procedure.

The adoption of thoria-promoted nickel methanation catalysts was instantaneous, being immediately acknowledged as superior by ter Meulen (52) and being used by practically all of the experimenters working with the method with only one or two notable exceptions. Using thoria-promoted nickel, Lacourt (36) reported the results on the determination of oxygen in eighteen compounds, some

of which contained nitrogen and sulfur, to be accurate to 0.2 absolute per cent, on the average. With this catalyst, all of the oxygen in the sample finally appears as water (34, 73). Newer methods of preparing and regenerating the catalyst have improved its life and efficiency, largely eliminating the earlier disadvantages of tedious preparation and ready susceptibility to poisoning. Russell and Fulton (73), in their publication describing the use of thoria-promoted nickel as methanation catalyst, also suggested the use of platinized quartz as the cracking surface to replace the platinized or plain asbestos heretofore used.

In 1935 Gauthier (22) used pumice as the cracking surface and nickelized pumice as the hydrogenation catalyst.

An interesting development in the hydrogenation technic is due to Goodloe and Frazer (25), who proposed the use of nickel chromite for both cracking agent and hydrogenation catalyst. They found that this substance is capable of absorbing a large amount of sulfur without a serious impairment of efficiency. A further advantage claimed for it was that it yields only elemental nitrogen from compounds containing nitrogen, eliminating the necessity for the absorption of ammonia from the gases issuing from the hydrogenation tube. Analyses of 45- to 135-mg. samples were successfully carried out in 1 hr. In a laboratory manual for quantitative organic analysis, Shriner (84) gives Goodloe and Frazer's procedure for the use of students. Shriner (85) states that lengthy conditioning of the nickel chromite catalyst is required, and that experience is necessary in order to obtain consistently good checks. Beginning students frequently get very erratic results, but with practice results accurate to 1 relative per cent may be expected.

In 1937 Kirner (34) published a thorough study of the micro ter Meulen method, using 200-mesh nickel gauze heated to 1000°C. for pyrolyzing the sample and thoria-promoted nickel and nickel wire as hydrogenation catalyst. The experimental set-up is illustrated in figure 2. The conventional Pregl purification train, A to E, is employed, except that platinized asbestos heated to 400°C. is used in the preheater D. Beginning at the exit end, the combustion tube contains an asbestos resistance plug (2 mm. long), 3 cm. of compressed nickel wire, 14 cm. of 2 per cent thoria-nickel catalyst, and a 17-cm. roll of 200-mesh nickel gauze. The mean accuracy of the results on six compounds containing only carbon, hydrogen, and oxygen was ± 0.10 per cent. Kirner found it necessary to deduct an empirical blank which is less than the blank actually determined; this was interpreted as indicating a hidden compensating error in the method. Low results and a voluminous deposit of carbon were obtained in the analysis of sucrose; this difficulty with polyhydroxy compounds has been reported by others. The topics of the blank value, sucrose analysis, and regeneration of the catalyst are discussed in some detail.

Unterzaucher and Bürger (94, 95) also described a microanalytical method for the determination of oxygen in organic compounds which is applicable to compounds containing nitrogen and sulfur, yielding good results on 3- to 5-mg. samples. Their later work (95) is worthy of mention, since they critically studied many of the factors involved.

In the most recent micro procedure published, Bürger (12) used a quartz tube, 60 cm. long and 0.8 cm. in diameter, connected at the inlet end to an electrically heated copper spiral and a tube of phosphorus pentoxide for purification of the hydrogen, and at the exit end to a water absorber and a Mariotte bottle for regulating the gas flow. The tube filling, beginning at the exit end, consists of (1) a small wad of silver wool, (2) 18 cm. of powdered nickel formate containing 10 per cent thoria (reduced *in situ*), (3) 3 cm. of silver wool, (4) 4 cm. of nickel spiral, (5) a thin layer of silver wool, (6) 2 cm. of very pure granulated calcium oxide, and (7) 16 cm. of quartz glass wool.

The cracking surface is heated to 1100°C. and the contact material to 300°C. The procedure is essentially as follows: (1) a 3- to 5-mg. sample in a platinum boat is inserted in the combustion tube, (2) the tube is swept from the exit end

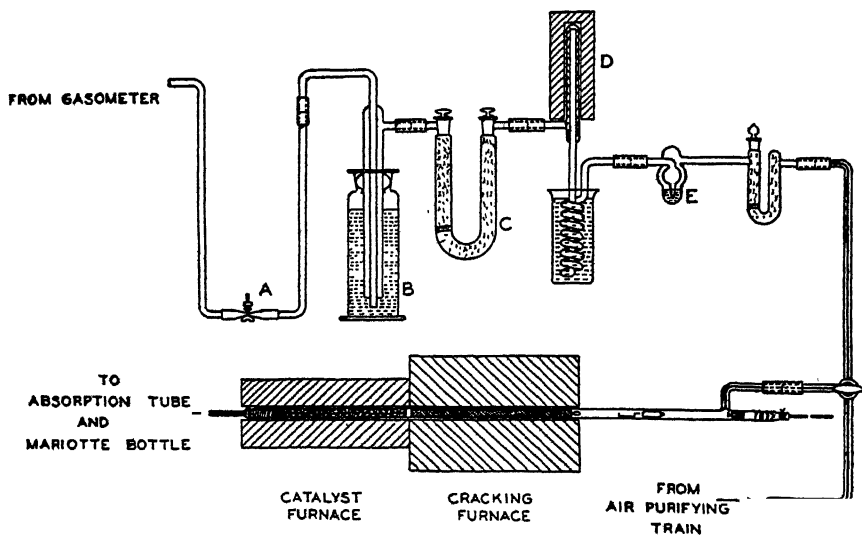


FIG. 2. Apparatus for the determination of oxygen by hydrogenation (from Kirner (34))

with hydrogen for 5 min. to displace air, (3) the absorber and Mariotte bottle are connected to the combustion tube, (4) the hydrogen flow is regulated to 8 ml. per minute, (5) the sample is volatilized with a hot flame, and (6), after the passage of 100–200 ml. of hydrogen, the water absorber is weighed.

Compounds containing nitrogen, sulfur, or halogens can be analyzed, although a separate determination of the halogen content is necessary. The use of calcium oxide, nickel, and silver in removal of interfering elements will be discussed in a subsequent section.

Lacourt (37, 38, 39) has done some interesting work on the micro hydrogenation method for determining oxygen in organic compounds. In an early publication she found an accuracy of ± 0.2 per cent on analyzing twenty-one compounds (37), but claimed an accuracy of better than ± 0.1 per cent in a later paper using an improved form of the method (38). The analysis of twenty

samples of succinic acid for oxygen gave results varying from 54.20 to 54.40 per cent, with an average value of 54.24 per cent, as compared to a calculated oxygen content of 54.31 per cent.

In 1941 the results of a critical study of the hydrogenation method were published in three articles by Morikawa, Kimoto, and Abe (66, 67, 68). The study was originally undertaken to ascertain the reason for the success of Abe's technic (29) with sucrose, which Kirner (34) and others had been unable to analyze successfully by the ter Meulen hydrogenation method. Using a platinum-silica catalyst (66), excellent results were obtained if certain critical factors were controlled. A cracking temperature of 950°C. is necessary, 200°C. higher than originally used for platinized quartz (73); the reducing temperature must be about 350°C., poor results being obtained if the temperature is below 300°C. or above 400°C.

The mechanism of the reaction on each of the two catalysts was thoroughly investigated. The factors studied in connection with the mechanism of cracking on the platinum-silica gel catalyst (66) included: (1) rate of flow of hydrogen, (2) rate of heating sample, (3) influence of temperature on the products of cracking, (4) reaction between carbon and carbon dioxide, (5) reaction between carbon and water (steam), (6) reaction between carbon and hydrogen, and (7) reaction between carbon dioxide and hydrogen. The hydrogenation catalyst studied was nickel containing 2 per cent thoria (67). A hydrogen flow of 5 liters per hour was used.

The following conclusions were drawn from experiments and theoretical considerations: At 800–900°C. all organic compounds containing oxygen decompose, forming carbon monoxide, carbon dioxide, and water. The higher the cracking temperature, the greater is the relative amount of carbon monoxide formed; rapid heating of the sample favors carbon dioxide formation. As the rate of flow of hydrogen increases, less carbon dioxide and more carbon monoxide are formed, while the water remains practically constant. A high percentage of oxygen in the compound favors carbon dioxide formation. The optimum temperature for reduction of carbon monoxide and dioxide on the nickel-thoria catalyst is 350°C. Above 400°C. the reduction of carbon dioxide may be incomplete. Too rapid cracking of the sample or a decrease in the concentration of hydrogen relative to that of the carbon oxides increases the amount of unreduced carbon oxides. Ethane and ethylene formed during cracking are converted to methane on the nickel-thoria catalyst. Hydrogen sulfide poisons the catalyst and should be removed between cracking and reduction. Morikawa, Kimoto, and Abe (68) applied the knowledge collected in regard to the hydrogenation method to a study of the decomposition of sucrose, cellulose, lignin, and coal by hydrogenation at various temperatures, which enabled them to indicate the relative stability of different types of oxygen bonds.

In a significant study (44), Marks applied the method to the determination of oxygen in oxidized oils, obtaining a precision of ± 0.1 per cent or better and an expected accuracy compared to calibration determinations of ± 0.2 per cent or better.

In general, aliphatic compounds are readily cracked to gaseous products between 600 and 800°C. with little carbon deposition, thereby maintaining the efficacy of the cracking surface (74). The deposition of considerable amounts of carbon by aromatic compounds which may require cracking temperatures up to 1100°C. results in rapidly diminishing effectiveness of the cracking surface.

The need for efficient and complete decomposition has also been pointed out by Gauthier (22), who warned of the danger of solid or liquid samples or their decomposition products distilling into the water absorber.

Successful use of the method depends to a considerable extent upon vaporizing the sample very slowly and uniformly over a period of time, e.g., 30 min., when using samples of 0.1 to 0.2 g. (55, 73).

A relatively high rate of hydrogen flow has been advocated because this tends to prevent deposition of carbon on the catalyst and decreases the sweeping time involved. For the macro method, flows of 90 ml. per minute (73) have been used, although 5 ml. of hydrogen per minute has been found satisfactory for the micro technic (34). The purification of the hydrogen used in the ter Meulen method has been critically discussed by Lindner and Eickhoff (43).

(a) Presence of elements in addition to carbon, hydrogen, and oxygen

As was previously mentioned, the presence in the sample of elements other than carbon, hydrogen, and oxygen complicates the determination, owing to the formation of hydrogen derivatives of these additional elements, e.g., ammonia, hydrogen sulfide, and hydrogen halides. In the analysis of samples containing these elements, the interfering hydrides can be removed prior to the water absorption or the amount of interfering hydride absorbed by the desiccant can be determined and the oxygen value suitably corrected. If the hydride of the nitrogen, sulfur, or halogen is formed quantitatively, the independent determination of the hydride in the desiccant permits the simultaneous determination of the oxygen and the additional element. Other possibilities are the use of catalysts or agents which prevent the formation of the undesired hydrides, or of desiccants which do not absorb the hydrides other than water.

Compounds containing nitrogen yield both elemental nitrogen and ammonia in the cracking and hydrogenation processes. Since the ammonia formed would be absorbed in the simple calcium chloride tube used in the method as first proposed, leading to erroneous results for oxygen, ter Meulen (48) recommended that a special absorption tube be used, one part containing dilute sulfuric acid and the other part containing calcium chloride. The ammonia is absorbed in the sulfuric acid and determined by back titration, the amount found being subtracted from the increase in weight of the absorption tube to give the increase due to the water alone. A similar procedure was suggested for halogen compounds, with a silver nitrate solution in the absorption tube to retain halogen acids. Russell and Marks (74) proposed the use of pellets of sodium hydroxide as absorbent for the water in the analysis of nitrogen-containing compounds, because this reagent absorbs no ammonia. Similarly, freshly ignited calcium oxide has been recommended (94).

Russell and Marks (74) report that 33 to 85 per cent of the nitrogen in representative organic compounds is converted to ammonia when platinized quartz the thoria-nickel catalysts are used, whereas no ammonia is formed when the nickel chromite catalyst is used (25).

Linder and Wirth (42) and others have used calcium oxide in the hydrogenation tube to absorb the hydrogen halides formed in the analysis of halogen-containing compounds. The reaction of the hydrogen halides with the calcium oxide produces an equivalent amount of water, which is absorbed by the desiccant.



This method, accordingly, necessitates a separate determination of the halogen present in order to correct the oxygen value, which is calculated from the weight of water absorbed.

A method claimed to be better than the calcium oxide technic consists in combining the hydrogen halides with ammonia and condensing the product in a cool section of the combustion tube (12). Another method suggested (48) consists in inserting a known quantity of silver nitrate at 120°C. between the two catalysts and determining the excess silver nitrate titrimetrically, from which a suitable correction can be applied to the weight of water found. Goodloe and Frazer (25) used a short layer of silver sulfate at 111°C., preceding the water absorber, to remove halogens.

A solution containing silver sulfate and sulfuric acid, located in the fore part of the water absorber, has been utilized (48) in the analysis of compounds containing halogen with or without nitrogen. The excess silver ion was determined by thiocyanate titration, and the ammonia was distilled from the solution after alkalization and was titrated. Good results for oxygen were obtained even in the analysis of chloranil, which contains 57.4 per cent chlorine.

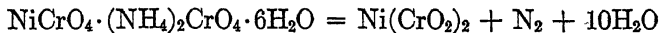
Unterzaucher and Bürger (95) employed a 3-cm. roll of nickel gauze and a layer of calcium oxide, heated at 600–700°C., between the cracking catalyst and the hydrogenation catalyst. The nickel gauze retains sulfur, and the calcium oxide reacts with the halogen acids as previously described.

The removal of sulfur is highly desirable, since it is retained by the cracking surface and poisons the nickel hydrogenation catalyst in a single run (29). In the analysis of coal and other organic materials, sulfur has been removed by a layer of nickel oxide, NiO, located between the cracking surface and the hydrogenation catalyst (29). A layer of finely divided nickel at 150°C., inserted after the cracking agent, effectively removes sulfur (48); this material maintained at 125°C. will remove sulfur, halogen, or both, but removal of the halogen may not be complete. The thoria-promoted nickel catalyst seems to be more resistant than unpromoted nickel to poisoning by the hydrogen sulfide formed in the pyrolysis and reduction of sulfur-containing compounds in the presence of hydrogen. Bürger (12) used silver wool to remove sulfur from the exit gases.

(b) Preparation of catalysts

The cracking surface can be prepared by ignition of asbestos treated with 10 per cent platinic chloride solution (2), or by ignition of 20-mesh granulated quartz with 5 per cent ammonium chloride and enough 10 per cent platinum chloride to wet the mass (73). Kirner (34) used merely a roll of 200-mesh nickel gauze, while the Japanese workers have used platinized silica gel (29, 67).

The nickel chromite catalyst proposed by Goodloe and Fraser (25) is prepared by reduction of nickel ammonium chromate on heating at 200°C.



Directions for the ready preparation of the double salt are given by Goodloe and Fraser.

Nickel alone is not adequate to accomplish efficiently the quantitative reduction of the carbon oxides to methane and water. When used a long time, even a highly active nickel catalyst gives progressively decreasing values for oxygen (95). ter Meulen's nickelized asbestos catalyst had to be removed after every determination, the carbon deposits burned out in a stream of air, and the mixture of nickel oxide and asbestos replaced in the tube and reduced (55). The efficiency of the catalyst can be maintained by the addition of promoters such as the so-called water-forming catalysts, e.g., the difficultly reducible aluminum, titanium, and thorium oxides. The most satisfactory methanation, hydrogenation, or reduction catalyst is active nickel containing 2 to 10 per cent of thoria. To make such a catalyst (73, 95), a mixture of the nickel and thorium oxides is prepared by heating the nitrates or formates either in or out of the combustion tube. The mixture is reduced *in situ* with hydrogen. The initial reduction at 300–400°C. usually requires a period of 48–72 hr., while regenerative reductions can usually be done overnight at 400°C. (73). Russell and coworkers (73, 75) recommended regenerating the catalyst by first oxidizing it to remove deposited carbon and then reducing it. It has been claimed that the catalyst is rendered more resistant to poisoning by sulfur if it is reduced at 450°C. instead of at 400°C. (75).

For the analysis of compounds containing sulfur, it has been recommended that a thoria-promoted nickel catalyst supported on granular quartz be used (75). The thin layers of nickel present are readily activated by reduction in hydrogen overnight at 500°C.

ter Meulen (55) advised the use of iron-free nickel oxide in preparing the catalyst, since iron slows up the rate of reduction.

(c) Determination of water formed

Various substances have been proposed and used as absorption agents for the water produced in the reaction, including calcium chloride (29, 48, 50, 73), calcium oxide (95), calcium sulfate (2, 25, 34, 37, 44), lithium hydroxide (95), magnesium perchlorate (37), and sodium hydroxide (29, 74). These, of course, involve the gravimetric determination of the water formed.

When an unpromoted or unactivated nickel catalyst is used for the hydrogenation process, as was true of all or most of the work prior to that of Russell and Fulton (73), not all of the oxygen present is converted to water. This necessitates the use of an absorbent for carbon dioxide, following the water absorbent, for the determination of any carbon dioxide present in the exit gases.

Lindner and Wirth (42) used the hydrogenation procedure with a titrimetric determination of the water formed. The exit gases are brought into contact with naphthyldichlorophosphine oxide ($C_{10}H_7POCl_2$, "phosphin"), which reacts with any water present to give hydrogen chloride. The latter is then absorbed and determined by titration with standard base. Lacourt (38, 39), apparently independently, suggested a similar titrimetric method. The hydrogenation products are bubbled through cinnamoyl chloride, the water reacting with the acid chloride to give cinnamic and hydrochloric acids, which are absorbed in alkaline solution.

To conclude and summarize the discussion of the catalytic hydrogenation or ter Meulen method for determining oxygen in organic compounds, the main advantage of this method is that it gives a direct and independent determination of the oxygen present in the compound, without the necessity of a knowledge of the carbon and hydrogen present. If, however, halogen is present, its determination is usually also necessary.

The disadvantages of the method are the complications introduced by the presence of elements other than carbon, hydrogen, and oxygen; the poisoning of the catalyst by sulfur and halogen, resulting in decreased activity and the need for frequent renewal; the voluminous deposition of carbon in the tube by many or most compounds; and the fact that the factor for oxygen ($O/H_2O = 0.88$) is unfavorable. Some workers report that a more or less empirical blank is necessary, and almost all workers have difficulties with certain polyhydroxy compounds, particularly sucrose.

2. Miscellaneous hydrogenation methods

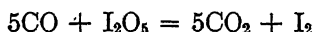
Sadtler (77) reported the determination of oxygen in asphalt by reducing the material with hydrogen in the presence of iron wool at a high temperature, converting the oxygen to water. Sulfur is retained by the iron. Any hydrocarbons are condensed out of the resulting gases before the water vapor is absorbed in a calcium chloride tube. The assumption is made that there is complete reaction to give water as the only oxygen-containing product, which is probably not what actually occurs. The method was applied only to asphalt samples and no pure compounds were analyzed to check the method.

Reduction with hydrogen over finely divided carbon at white heat was suggested by Boswell (8). The products of the reaction are water, carbon dioxide, and carbon monoxide. The water is absorbed in sulfuric acid, the carbon dioxide is absorbed in soda lime, and the carbon monoxide is oxidized to carbon dioxide with iodine pentoxide at $170^\circ C$. and is then absorbed in soda lime. The results of single analyses of five compounds are presented, the average error being 0.3 absolute per cent. The chief faults of this method lie in the facts that three

oxygen-containing products must be determined, thus increasing the chances for error, and that no provision is made for compounds containing elements other than carbon, hydrogen, and oxygen. Ten weighings of absorption tubes are required for each analysis.

C. THERMAL DECOMPOSITION OVER CARBON

A new attack on the problem of determining oxygen in organic compounds was recently proposed by Schütze (78, 79, 80), who described a semimicro method which he stated had been in use for several years (since 1936) with very successful results. In this procedure the sample is thermally decomposed in a stream of nitrogen and the cracked products are led over carbon at 1000°C., resulting in the formation of carbon monoxide. The carbon monoxide is then oxidized at room temperature with iodine pentoxide, yielding carbon dioxide and iodine.



Either the carbon dioxide or the iodine may be determined: the former by absorption in Ascarite, or the latter by titration with standard thiosulfate solution.

The time required for an analysis is given as 1 hr. The results of the analysis of five compounds, including compounds containing sulfur, halogen, and nitrogen, were accurate to ± 0.2 per cent or better for oxygen contents of 13 to 34 per cent.

At the same time, an adaptation of Schütze's method to microchemical apparatus was made by Zimmermann (99). Using 5-mg. samples, Zimmermann obtained results accurate to 1 relative per cent of the oxygen content, e.g., 26.40 per cent instead of 26.21 per cent, with compounds containing nitrogen, sulfur, and halogen in addition to carbon, hydrogen, and oxygen. However, it was necessary to use rather large blank values, e.g., 100 γ of carbon dioxide. Apparently the large blank values were caused by air trapped in the apparatus, including the sample boat, during the process of adding the sample and removing the combustion boat after the analysis. This effect, which is of great importance in the determination of oxygen, has also been discussed by Kirner (34). Zimmermann claimed that the apparatus was suitable for automatic combustion and he indicated how several sets of apparatus could be handled simultaneously.

Unterzaucher (93) in 1940 made certain improvements in Zimmermann's apparatus and procedure which resulted in the elimination of the large blanks mentioned. In addition to the determination of oxygen in pure compounds, he was interested in the determination of small amounts of oxygen such as might be present in impurities like oxidation products. Unterzaucher's apparatus is shown in figure 3.

The apparatus from Nos. 1 to 10 inclusive is designed to supply nitrogen at constant pressure and to purify it by the removal of any oxygen that might be present. A quartz combustion tube is used with a 12-cm. layer (18) of small carbon particles electrically heated to 1120°C. No. 16 is a small electric furnace moved by a synchronous motor and used to burn the sample in the platinum boat (17). Coarse-grained potassium hydroxide (21) is employed to remove sulfur, halogen, and nitrogen compounds. The iodine pentoxide in No. 22 is heated by

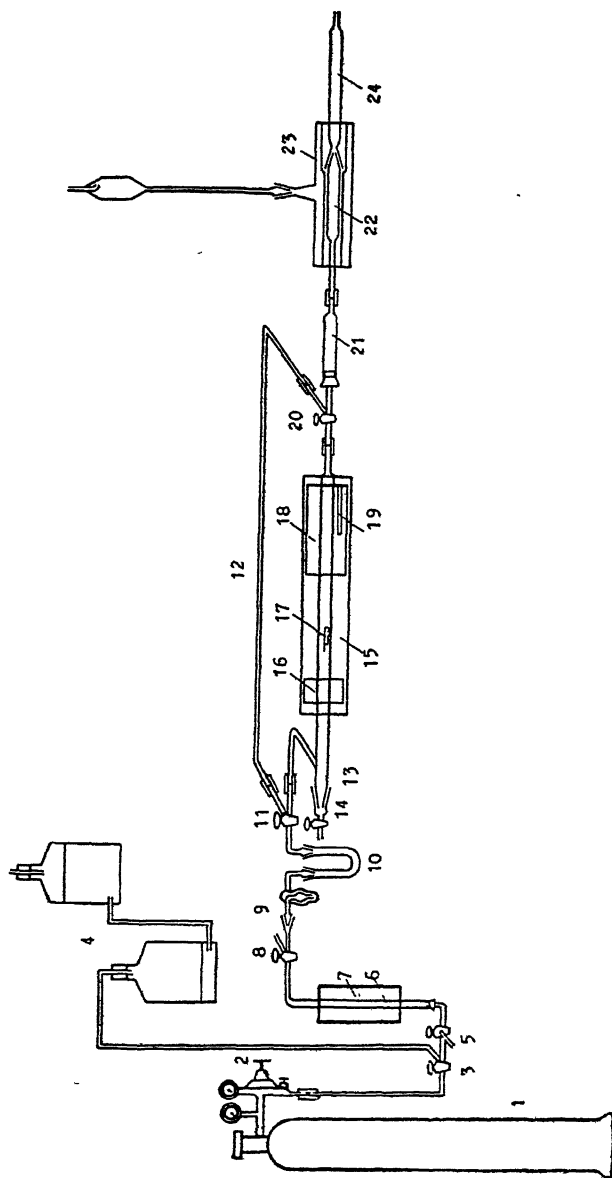


FIG. 3. Apparatus for the determination of oxygen by thermal decomposition over carbon (from Unterzaucher (98))

refluxing glacial acetic acid in the mortar (23). The sides of the absorption tube (24), which are indented to increase their surface, are moistened with 20 per cent sodium hydroxide solution. The principal innovation in the apparatus is the flushing tube (12) by use of which, in conjunction with the proper stopcocks (11, 14, 20), the air can be very effectively and simply removed from the combustion tube after the sample has been inserted.

In Unterzaucher's procedure, the gas mixture resulting from thermal decomposition of the sample in oxygen-free nitrogen is passed over carbon heated to 1120°C., resulting in quantitative conversion of the oxygen to carbon monoxide. After the removal of interfering gases by solid potassium hydroxide, the carbon monoxide is oxidized to the dioxide by iodine pentoxide heated to 118°C. The iodine produced is volatilized out of the iodine pentoxide tube, absorbed in dilute sodium hydroxide, and oxidized to iodate with bromine. After the addition of potassium iodide solution and acidification, the iodine formed is titrated with 0.02 *N* sodium thiosulfate solution. Since 1.000 ml. of 0.02000 *N* thiosulfate solution is equivalent to only 0.1333 mg. of oxygen, the procedure is suitable for the accurate measurement of small amounts of oxygen.

The most effective carbon found by Unterzaucher was lampblack-type carbon obtained from I. G. Farbenindustrie A.-G. which was moistened, dried, cut into small cubes, and ignited in nitrogen. Another effective manner of preparing the reduction medium is to press the wet paste of carbon through a glass nozzle of 1-mm. diameter and to crush the resulting threads after they have been dried and ignited in nitrogen. Experiments show that it is possible to prepare a satisfactory carbon by the cracking of a suitable organic substance in nitrogen. In general, a single carbon filling suffices for the life of the combustion tube.

The sample weight commonly is 3 to 4 mg., although in compounds of very low oxygen content a 20- to 30-mg. sample may be used; samples as large as 100 mg. have been employed when determining oxygen due to an impurity. The flow of nitrogen is regulated to 8-12 ml. per minute, 300 ml. being used in each determination. An analysis requires 30 min.

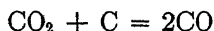
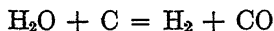
The samples analyzed included solids and liquids containing nitrogen, halogen, and sulfur in addition to carbon, hydrogen, and oxygen. The amount of oxygen in the sample and the type of linkage have no effect. The determination of oxygen in amounts of 0.1 to 0.7 per cent in oxygen-free compounds to which minute amounts of oxygenated compounds were added was satisfactory.

The necessity of using a high temperature was indicated by a series of determinations on pure substances at various temperatures.

Unterzaucher claimed that in many cases this method, as used during 1939 in the I. G. laboratories, was superior to the usual methods of elementary analysis. The analysis of twenty-six compounds gave results for oxygen differing from the theoretical values by an average of only 0.1 per cent.

Korshun (35) tested Schütze's procedure with pure organic compounds, checking the final step by determining both the carbon dioxide and the iodine formed. The liberated iodine was absorbed by activated copper and the carbon dioxide by Ascarite. A single determination required 30 to 40 min.

The principal disadvantage of the method involving reduction over carbon to carbon monoxide is the high temperature needed. A high temperature is required because fundamentally this method involves the water gas reactions of reducing water and carbon dioxide by contact with carbon at high temperatures.



These reactions necessitate the use of high temperatures. The work cited (93), unpublished experiments (21), and available thermodynamic data indicate that a temperature exceeding 1000°C. and probably near 1100°C. is necessary for substantially complete conversion to carbon monoxide under the necessarily dynamic conditions of the method.

The advantages of the method are very real when compared to the other two technics,—complete combustion and catalytic hydrogenation,—which have been extensively investigated for the determination of oxygen in organic compounds. The apparatus is simpler than that required for complete combustion in oxygen and no complicated calculations are necessary. There is no catalyst to be poisoned as in the ter Meulen method, and any carbon formed by pyrolysis of the compound usually serves to increase the contact mass for the conversion of oxygen to carbon monoxide. The presence of elements other than carbon, hydrogen, and oxygen apparently has no effect on the applicability of the method. Furthermore, the factor used to calculate the oxygen ($\text{O}/\text{CO}_2 = 0.3636$) is very favorable. Inherently the method is simple, accurate, and dependable.

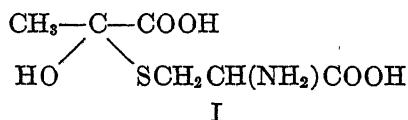
This method is probably the most promising attack on the problem of determining oxygen in organic compounds. Work which was in progress at Purdue University (21), and which was interrupted by the necessity of using the available manpower and apparatus on war problems, indicates the possibility of using a simpler experimental set-up than Unterzaucher employed (93), and a more active reducing medium in producing carbon monoxide than carbon alone.

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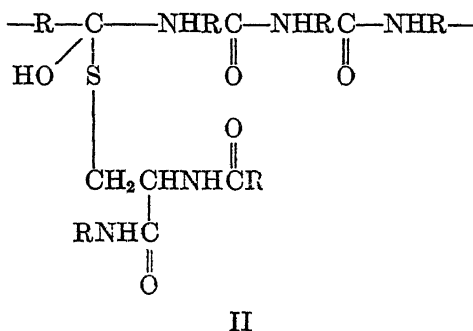
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tobacco mosaic virus protein in the native state, unreactive to nitroprusside, could be treated carefully with iodine, whereby the sulfhydryl groups of the protein were oxidized without loss of infective power of the virus. What known chemical groupings involving sulfhydryl are unreactive to nitroprusside but reactive to iodine?

Cysteine may combine with pyruvic acid to form an addition compound in which the sulfhydryl group residue reacts stoichiometrically with iodine, yielding disulfide, but does not react at all with nitroprusside (355). The structure of this hypothetical compound may be represented in formula I:

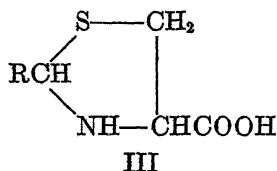


In proteins an analogous formulation may be tentatively considered, in which the sulfhydryl groups of the cysteine constituent of one polypeptide chain combine with the ketonic portion of a peptide linkage in an adjacent polypeptide chain as in formula II:



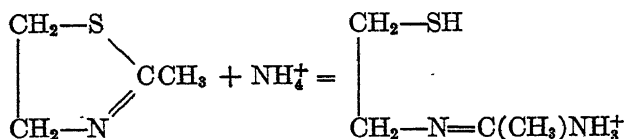
The critical test for the possible validity of formula II would be to observe whether the weak binding between ketone and sulfhydryl could be dissolved by solutions of guanidine salts or related compounds.

Cysteine may also condense with aldehydes, with the loss of a molecule of water, to form thiazolidines (335, 355), as in formula III:



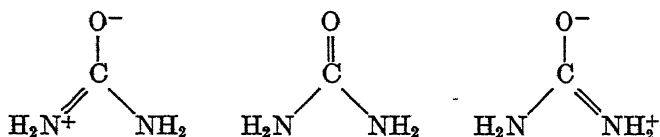
These compounds also react stoichiometrically with iodine and fail to give a nitroprusside test. The possibility that such ring structures, analogous to the oxazoline rings suggested earlier by Bergmann *et al.* (56, 59), may exist within the native protein structure was advanced by Linderstrøm-Lang. In an im-

portant paper (233), the latter demonstrated the essential lability of the thiazolidine ring toward ammonium salts, whereby the ring was opened in the presence of the latter, probably in the following manner:



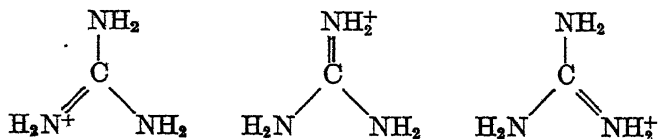
Furthermore, the ease of opening the ring was a function of the nature of the anion of the ammonium salt used and increased in the sequence sulfate, chloride, bromide. The same sequence was found in the effect of various guanidine salts in the liberation of sulfhydryl groups in egg albumin (162). The difficulty in applying these results to the problem of protein denaturation lies in the fact that the pure guanidine salts, and also urea, fail to open the thiazolidine ring. However, as Linderstrøm-Lang pointed out, only the simplest thiazolidine, the 2-methyl compound, was employed, and the further study of this effect would require the examination of more complex representatives of this class of substances.

The extent of the protein-denaturing effect which the various *N*-substituted ureas and guanidines exert has been related to the capacity of the latter to exist in several resonating structures which are in equilibrium with one another (160). Urea, for example, may be represented as resonating among the following three structures:



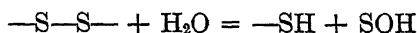
Replacement of hydrogen by methyl, as in *N*-methylurea, might conceivably tend to hinder the double bond from migrating to the methylated nitrogen, owing to the greater electronegativity of carbon over hydrogen. With asymmetric *N,N'*-dialkyl substitution this hindrance might be expected to be somewhat greater. A very much greater hindering effect would be expected for symmetrical *N,N'*-dialkyl substitution, for here the resonance of the double bond would be considerably restricted, and the neutral form (the center one above) would be much more important than the other two, charged, forms. The methylated ureas sometimes are weaker than urea in denaturing the proteins (table 4), and symmetrical *N,N'*-diethylurea possesses no denaturing effect at all (192).

The guanidinium ion resonates among the following structures:



Substitution of a methyl group in this ion produces no difference in the denaturing effect of the parent ion on certain proteins, and reduces this effect with other proteins (table 4). The *as*-dimethylguanidinium ion, in which resonance may be expected to be still more restricted, actually shows a distinctly smaller denaturing effect on the proteins than either the unsubstituted or the monosubstituted ions (table 4). An interesting series of experiments in harmony with this, as yet quite tentative, hypothesis has been described by Dickens (126). The latter observed that the Pasteur reaction in brain cortex could be inhibited by guanidine salts, and that the maximum inhibition was brought about by guanidine and methylguanidine, whereas *as*-dimethylguanidine produced little effect.

In the light of Anson's results (8) and of suggestive evidence from simpler molecules, the presence of preformed sulfhydryl groups within the native protein may be taken for granted. It is *surprising*, however, that many authors assume that the *sulfhydryl groups* which appear in the denatured protein owe their *origin* to hydrolytic fission of *disulfide linkages* (cf. 140), an assumption which probably had its origin in Hopkins' classic paper (192). The evidence against this assumption is considerable and may be given briefly as follows: (a) No synthetic peptide of cystine treated with solutions of urea or guanidine salts shows the presence of sulfhydryl; the S—S bond is completely inert to these protein denaturants (160). (b) Proteins such as amandin and insulin, the latter high in cystine, when denatured give no reactions for sulfhydryl groups. (c) All the non-methionine sulfur of myosin (168), and after denaturation of the proteins all the sulfur of the tobacco virus protein (346), can be accounted for in terms of cysteine; if the reaction were



as assumed, then only half instead of all the "cystine" sulfur could be obtained in these proteins as cysteine. (d) If disulfide groups were converted wholly or in part to sulfhydryl by protein-denaturing agents it would be quite impossible to oxidize quantitatively the sulfhydryl groups of the denatured protein to disulfide groups in the presence of the denaturing agent; since this procedure is now commonly used, the suggestion is of course untenable.

B. THE KINETICS AND THERMODYNAMICS OF PROTEIN DENATURATION

Study of the kinetics of denaturation may give indirect evidence for protein structure as well as for the mechanism of denaturation. From the experimental determination of the order of the reaction one may hope to distinguish whether denaturation is an intramolecular or an intermolecular transformation. From the magnitude of the classical energy of activation or critical increment, or, in terms of the newer theory of absolute reaction rates, from the free energy of activation and the entropy of activation, one may estimate the number and nature of the bonds broken in the disruption of the specific native configuration. This information may then be used in the derivation of a theory of denaturation or in a test of existing theories.

However, this prospect may not be fully realized as yet because of the paucity of the data now available and because of the consistent occurrence of kinetic anomalies shortly to be described.

1. *Experimental methods for following the course of the reaction*

The familiar example of heat coagulation early led to the study of the effect of temperature on proteins. It was long believed that the temperature of heat coagulation, or, in the case of biologically active proteins, the temperature of heat inactivation, though somewhat influenced by ionic environment, was a physical constant characteristic of the particular protein. However, in view of later findings that heat coagulation and inactivation are measurable and not instantaneous processes, the voluminous literature on this subject may be disregarded. It will be shown later that the apparent existence of a critical temperature of denaturation arises from the high temperature coefficient characteristic of the reaction.

The discernment of the various processes involved in heat coagulation may be ascribed to Hardy (175), who first advanced the now generally accepted view that heat coagulation consists of two distinct stages. In modern terminology these are (1) the initial thermal denaturation,—conforming to the definition already proposed in this review; (2) the actual coagulation,—a colloidal phenomenon less significant for the study of the mechanism of denaturation.

Other modes of denaturation may likewise involve a subsequent step of coagulation, e.g., surface denaturation (78) and denaturation by ultraviolet irradiation (107). However, coagulation may be averted by the use of denaturants which exert a dispersive action, for example, urea and guanidine hydrochloride. Confusion has evolved from the fact that the term "coagulation", when applied to proteins, has often been used to signify both the precipitation and the preliminary denaturation.

In the measurement of the reaction kinetics of protein denaturation it is important to isolate the actual process of denaturation so that its velocity is the rate-determining step of the over-all reaction. Yet, since the appearance of a coagulum is the most tangible evidence of denaturation, the measurement of the rate of formation of insoluble protein (or conversely, the rate of disappearance of soluble protein) has been used commonly to describe the kinetics. Under conditions which facilitate rapid flocculation, that is, in the presence of a small amount of neutral salt, the initial reaction of actual *denaturation* may assume the rate-determining rôle, so that the concentration of residual protein in the supernatant solution becomes equivalent to that of unchanged protein.

This was the experimental method introduced by Chick and Martin (96–100) and commonly adopted by subsequent workers. Turbidimetric measurement of the salt-free protein denatured at its isoelectric point is an analogous procedure, in that flocculation proceeds most rapidly at the isoelectric point but differs in the empirical nature of the method of measurement of the quantity of insoluble protein (107). Certain other methods of following the course of the reaction, for example, the notation only of the apparent time of first formation of a visible precipitate (148), at best may be considered only semiquantitative.

The measurement of the change in any singular property of a protein, such as the inactivation of an enzyme or virus, affords another criterion for observing the course of reaction. The residual activity may be taken as directly proportional to the amount of unchanged protein. Where reversal of inactivation is claimed, appropriate steps may be taken to halt this process. While it is true that the thermal inactivation of a virus may proceed at a different rate from the thermal change in other properties of the same substance (223), it is unnecessary to review again here our brief that denaturation may consist of a series of concomitant changes any one of which may be used as a criterion. Furthermore, the fact that protein denaturation and enzyme inactivation possess in common a high energy of activation evinces their relationship.

2. The order of the reaction

As a result of the classical experiments of Chick and Martin (96-100) the belief that heat denaturation is a first-order reaction has become firmly implanted in the literature and has been extensively drawn upon as support for the thesis of the intramolecular character of protein denaturation. However, it is now opportune to review this question.

It will be recalled that in a first-order reaction the rate is directly proportional to the concentration of the reacting substance (149). Similarly, in a reaction of the second order the rate is proportional to the product of two concentration terms, or to the square of one, and in a zero-order reaction it is independent of the concentration. Thus, the *order* is given by the number of molecules whose concentrations determine the speed of the process. The *molecularity*, however, refers to the number of molecules taking part in the act leading to chemical reaction. The order and molecularity frequently prove to be identical, but, for clarity, reactions whose rate has been shown to be empirically dependent upon but one concentration term will be hereinafter designated as of the first order. Since these terms were once considered synonymous, protein denaturations following a first-order law have often been reported to be uni- or mono-molecular in nature.

With a few exceptions, protein denaturation and biological inactivation have been reported to be first-order processes. Already in 1895 Tammann (382) had found the heat destruction of emulsin, both in solution and as a dry powder, to be a first-order reaction between 60° and 75°C., the data for the temperature range 40-60°C., however, apparently deviating from this law. Colleagues of Arrhenius next reported that the heat inactivation of trypsin, pepsin, and rennet all followed a first-order law (23), as did the diminution in hemolytic activity of unpurified vibriolysin, tetanolysin, and goat serum (141).

The first rigorous kinetic studies of the heat denaturation of crystalline proteins were those of Chick and Martin (96-100), who concluded that the heat denaturation of hemoglobin and of egg albumin at constant hydrogen-ion concentration was a first-order reaction. For hemoglobin, this result has since been verified by Hartridge (178) and by Lewis (226). However, the results for egg albumin are not unambiguous, and in view of the importance subsequently attached to these experiments, the data merit review.

Protein denaturation

(a) *Egg albumin*: In their first paper Chick and Martin reported that "heat coagulation" of unbuffered egg albumin solutions did not conform to a simple logarithmic (first-order) law (96). After recalculation of their data, Sutherland (376) concluded that the reaction was of the second order. Chick and Martin (97) pointed out that this was true only if the time of placing the egg albumin solution into the thermostat was taken as the initial time, the period of warming up being neglected. They then showed that in a solution of constant acidity (saturated boric acid) (97) or constant alkalinity (saturated magnesium oxide) (98) the heat denaturation of egg albumin was satisfactorily a first-order reaction. However, in unbuffered solutions in the presence of concentrated salts, denaturation "proceeded in extraordinary agreement with the progress of a reaction of the second order" (97). This behavior was considered adventitious and attributed to changing hydrogen-ion concentration.⁴ Subsequently, Lewis (227) found that a steady first-order constant was obtained in buffered solutions but, since the rate was much enhanced, reported only observations for unbuffered solutions for which a first-order velocity "constant" was obtained by a method of approximation.

It should be pointed out that this ambiguity with regard to the order of reaction for the heat denaturation of egg albumin is reflected in calculations of the critical increment and of the free energy and entropy of activation. For example, in the heat denaturation of egg albumin in 2 *M* ammonium sulfate (97), second-order constants (which exhibit excellent agreement and were used by Chick and Martin in the calculation of the temperature coefficient) yield a value for the energy of activation 19,000 calories greater (333) than that apparently obtained by the utilization of unsteady first-order constants (140). A similar criticism applies to the calculations of Lewis (227).

(b) *Hemoglobin*: Despite the ambiguity which surrounds the rate law governing the classical heat denaturation of egg albumin, it must be acknowledged that hemoglobin denaturation appears to obey a first-order law. This is true not only of heat denaturation, but holds also for the alcohol denaturation of hemoglobin (68), and apparently for the acid denaturation of the same protein (121). In contrast to the results reported for egg albumin (97), the presence of concentrated salts does not appear to change the order of reaction in the heat denaturation of hemoglobin (228).

(c) *Other proteins*: However, the denaturation of other proteins is not uniformly a first-order reaction. The heat denaturation of leucosin is of the first order (243), and it has been stated that the heat denaturation of insulin is likewise of the first order (148), although the velocity constant was observed to fall off for a sample of even thrice-crystallized protein. On the other hand, the well-investigated kinetics of the acid denaturation of crystalline edestin (39) may be described by either (a) an equation representing a reaction of high order or (b)

⁴ The denaturation of egg albumin by sodium hydroxide at 25°C. follows a "roughly bimolecular course," the pH falling steadily during the reaction (87).

the existence of two or more simultaneous unimolecular (i.e., first-order) reactions. In fact, an empirical equation was derived which expressed the rate of formation of edestan, the product of denaturation, as a function of the rates of four independent and simultaneous exponential reactions.

Biological inactivation

A first-order law is valid for the heat inactivation of a number of enzymes, e.g., pepsin (23, 231, 304), trypsin (23, 302, 311), pancreatic lipase (251), emulsin (382), and peroxidase (418). However, in the heat inactivation of malt amylase the course of reaction is certainly not of the first-order type, and though second-order constants are more satisfactory, they likewise show deviations (244). Similarly, the heat inactivation of invertase is not of the first order, the constants falling off to one-half the initial value even in phosphate-buffered solutions (139). Furthermore, while the heat inactivation of the plant proteases papain and bromelin follows a first-order law (bromelin, however, deviating at higher temperatures), the heat inactivation of asclepain (the protease of the milkweed *Asclepias speciosa*) conforms to a second-order equation (407). The admitted presence of impurities in many of these preparations entails doubt both as to the true order of the reaction and as to the validity of calculations of activation energies by the use of unsteady rate constants. It is therefore of interest to compare the kinetics of denaturation and biological activation in the case of several well-characterized proteins.

(a) *Pepsin*: The heat inactivation of crystalline pepsin is of the first order (241, 304). This is also true of the inactivation of crystalline pepsin by ultra-violet radiation (306) and of crude pepsin by the beta radiation of radium (195). Inactivation by weak alkali, first reported to follow a three-halves order (258) and then to consist of an instantaneous step followed by a first-order reaction (152), has recently been shown to be completely first order for crystalline pepsin (373).

(b) *Trypsin*: The heat inactivation of purified trypsin is evidently of the first order (302, 311), as is inactivation of the crude product by soft x-rays (103) and by the beta radiation of radium (195). However, between pH 2.0 and pH 9.0 Kunitz and Northrop found that the irreversible inactivation of crystalline trypsin obeys a second-order equation (214). This behavior was attributed to the existence of a simultaneous reaction involving the hydrolysis of denatured trypsin by active native trypsin. On the alkaline side of pH 13.0 and on the acid side of pH 2.0, the inactivation was found to be of the first order. Fair agreement with a first-order law was observed by Pace for the heat inactivation of various purified preparations of "trypsin" and associated enzymes (311, 312, 313, 314).

(c) *Tobacco mosaic virus*: Sufficient data are available for a comparison of the kinetics of thermal denaturation and urea denaturation only in the case of tobacco mosaic virus nucleoprotein. The measurements are complicated by the accumulation of nucleic acid in the supernatant solution as a result of the disintegration of virus protein. In the case of the thermal denaturation (223), for

the majority of the experiments there is little choice between a first-order or second-order or even a zero-order reaction, although the first-order equation is favored by statistical analysis of the data. However, in a critical experiment the error inherent in the accumulation of nucleic acid was averted by measurement of the amount of soluble protein capable of becoming insoluble on prolonged boiling. This method of analysis strongly favored a first-order law. An analogous experiment in the case of urea denaturation gave the same result (221). As in all experimental methods based upon an analysis of the residual soluble protein, this approach involves the tacit assumption that the rate-determining step is not the flocculation or, in this instance, the disintegration of the denatured protein. As yet unexplained is the fact that the reaction velocity was observed to vary inversely with the initial virus concentration.

The denaturation of the purified virus upon exposure to high pressure (7500 kilos per square centimeter) likewise appears to be a first-order reaction, although obedience to a zero-order equation has not been rigorously excluded (222).

It has been reported that the thermal inactivation rate of unpurified tobacco mosaic virus, as well as of several other viruses, follows the course of a first-order reaction (329). Inactivation appears to proceed at a considerably faster rate than thermal denaturation and is believed to be one of the earlier reactions in a series which leads to denaturation (223).

Two modes of denaturation the kinetics of which have been inadequately studied are ultraviolet irradiation and surface denaturation. The incidence of an initial step of light denaturation preparatory to the steps of temperature-dependent denaturation and flocculation has been postulated (106-108). The initial process is reported to be of the first order for egg albumin. Surface denaturation, however, appears to be a zero-order reaction, the rate being independent of protein concentration (78, 79, 412).

Critical analysis of the abundant literature on the kinetics of protein denaturation reveals that there are few studies which satisfy the minimal criteria justifying theoretical analysis: namely, (a) highly purified, preferably crystalline preparations as reactants; (b) adequate experimental methods. It is known that the presence of impurities may affect even the order of reaction (251, 302, 407), a fact not recognized by many early investigators. The justification for the second criterion is self-evident.

When the kinetic data are considered on the basis of the number of crystalline proteins subjected to various modes of denaturation and studied by rigorous experimental methods, it must be concluded that the denaturation of proteins is not unequivocally a first-order reaction.

It may be argued that most deviations from a first-order law may satisfactorily be explained by the assumption of the existence of simultaneous reactions or of other processes whose rates are not instantaneous relative to the denaturation reaction. Inasmuch as denaturation exhibits a procession of stages, a stepwise nature of the reaction mechanism may not be gainsaid. Each one of the steps subsequent to flocculation may be of the first order or, indeed, unimolecular, but, on the other hand, the experimental methods at our disposal do not enable us to

discern the kinetics of a given step, and for a kinetic description of the process one must still rely upon the empirical dependence of the reaction velocity upon the concentration of reaction substance, i.e., the order of the reaction.

3. The molecularity of the reaction

The *a priori* acceptance of a first-order law as the factor governing the kinetics of general protein denaturation has led to the assumption of a unimolecular process of denaturation involving an intramolecular change in the intrinsic configuration of the protein molecule. The validity of this hypothesis with regard to the data derived from non-kinetic sources will be discussed in Part III. While such a mechanism does seem to be in best accord with the facts now available, the significance of water and of the concentrations of hydrogen and hydroxyl ions should not be discounted. In fact, each of these three substances has been suggested as a reactant.

It is known that a dry preparation of egg albumin is not readily susceptible to heat denaturation (41, 63, 96). Indeed, a dry protein may be exposed to temperatures exceeding 120°C. without undergoing a change in solubility (96). This observation early led to the suggestion that denaturation was a reaction taking place between protein and water (96, 97). It is notable that the kinetics of the heat denaturation of dry egg albumin do not follow a first-order law (63). Unexplainably, this denaturation resembles an autocatalytic reaction.

In addition to the rôle of water, an extraordinary catalytic influence of hydrogen and hydroxyl ions on heat denaturation and enzyme inactivation has long been known. The great majority of such processes are highly sensitive to pH, a variation in pH of one unit often being accompanied by a hundredfold change in rate. In some instances the rate is strictly proportional to some inverse or direct power of the hydrogen-ion concentration, indicating that the formation of a charged species is requisite for activation.

4. The abnormalities of protein denaturation kinetics

The most striking characteristic of the kinetics of protein reactions is the extraordinary dependence of the rate of *thermal* denaturation upon temperature. At that temperature at which coagulation or inactivation is first observed (about 50°C.), the inception is so marked that there early arose a characterization of proteins in terms of "coagulation temperatures". Raising the temperature a few additional degrees may cause the velocity to become immeasurably fast. This fact precludes rate studies over a temperature range comparable to that employed in ordinary kinetics (about 70°C.), the temperature range in the case of thermal denaturations often being limited to but 2–3°C.

The effect of temperature upon rate may be expressed approximately by the *temperature coefficient*, Q_{10} , the ratio of the velocity constant at the temperature $t + 10^\circ$, to that at t° (i.e., k'_{t+10}/k'_t). Whereas, for ordinary processes Q_{10} is about 2 to 3, that is, the velocity doubles or trebles for each 10°C. rise, temperature coefficients in excess of 600 have been reported for protein denaturations (96). This abnormality has greatly stimulated the investigation of the kinetics

of protein denaturation, for such an effect of temperature may not be explained by known processes for the redistribution of energy between molecules.

A more satisfactory method of expressing the temperature dependence of reaction velocity is given by the Arrhenius equation:

$$\frac{d \ln k'}{dT} = \frac{E}{RT^2} \quad (1)$$

which upon integration over the temperatures T_1 and T_2 assumes the form

$$E = \frac{RT_2 T_1}{T_2 - T_1} \ln \frac{k'_2}{k'_1} \quad (2)$$

E is the *critical increment* or the *energy of activation*, k'_2 and k'_1 are the respective velocity constants,⁵ and R is the molar gas constant; the dimensions of E are calories per mole. Equation 2 indicates the existence of a logarithmic relationship between the temperature coefficient and the energy of activation. For reactions conforming to the ordinary laws of chemical kinetics the value of E at 50°C. is of the order of 20,000 calories ($Q_{10} = 2.6$). For protein denaturation and enzyme inactivation, values of E ranging in magnitude up to 198,000 calories (141) have been reported (cf. tables 7 and 8).

On the basis of the simple collision theory of kinetic activation it is not possible to explain the observable rates of reaction without the assumption of astronomical values of the steric factor. The latter quantity is introduced to accommodate the deviation from experiment and is supposed to allow for the specific orientations of the molecules necessary for a fruitful collision. For example, if the velocity constant be given by the expression

$$k' = PZe^{-E/RT} \quad (3)$$

(in which P is the steric factor, and Z the collision number), a magnitude of P of the order of 10^{72} (216) must be assumed in the thermal denaturation of egg albumin, for which E is reported to be of the order of 140,000 calories. This is in contrast to the ordinary value of P , which usually varies from unity to 10^{-8} , though higher values have been reported (149).

5. The theory of absolute reaction rates

Such exceedingly large energies of activation as those given in tables 7 and 8 and the correspondingly large temperature coefficients have stimulated interest in the mechanism of these reactions, and the resolution of the kinetic paradox has been attempted by two different methods of approach. The school endorsing the theory of absolute reaction rates has proposed (140) that the free energy of activation, $\Delta F^\ddagger$, rather than E , governs the rate of the reaction. In such a formulation, the steric factor or probability factor, P , is shown to involve an entropy term, and, indeed, formally P is related to $\Delta S^\ddagger$, the entropy of activa-

⁵ We use k' to denote the reaction velocity constant, since k , the Boltzmann constant, is introduced in the statistical definition of k' .

tion, for $P = e^{\Delta S^\ddagger/R}$ (216). Moreover, E , the critical increment or "experimental activation energy" obtained by substitution in the Arrhenius equation, is only approximately equal to the "heat of activation", $\Delta H^\ddagger$, for $\Delta H^\ddagger = E - RT$ (372). (At 50°C., RT equals approximately 640 calories.)

In place of the collision number, Z , a factor kT/h , having the dimensions of frequency, may be employed to represent the rate at which the activated molecules cross over the energy barrier (150). The quantity kT/h is a universal frequency dependent only on temperature, k being the Boltzmann constant and h being Planck's constant. The frequency factor PZ (equivalent to the constant A of the Arrhenius equation) is approximately equal to

$$\frac{kT}{h} e^{\Delta S^\ddagger/R}$$

the inequality arising from the fact that $\Delta H^\ddagger$ is not quite equivalent to the experimental activation energy, E .

The rate may now be expressed (140, 150) in terms of the thermodynamic functions $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta F^\ddagger$ (the transmission coefficient being taken as unity).

$$k' = \frac{kT}{h} e^{-\Delta F^\ddagger/RT} = \frac{kT}{h} e^{-\Delta H^\ddagger/RT} e^{\Delta S^\ddagger/R} \quad (4)$$

If the thermodynamic functions are known, this equation enables the calculation of the absolute rate of reaction. Conversely, the function $\Delta S^\ddagger$ may be calculated by substitution of the observed values of k' and $\Delta H^\ddagger$ (obtainable from E), $\Delta F^\ddagger$ may be derived by analogy to the well-known relationship $\Delta F = \Delta H - T\Delta S$.

From equation 4 it is apparent that the rate is governed not by the activation energy alone, but also by the entropy of activation. In other words, the free energy of activation is decisive in establishing the rate. For reactions characterized by abnormally high values of E , a measurable rate may still be attained if a correspondingly large entropy of activation is observed. Protein denaturation must accordingly be distinguished by large entropy changes (140), a conclusion inducing considerable speculation as to the nature of these changes, which are assumed to be intramolecular.

The importance of the application of this elegant statistical treatment to protein reactions is that a generalized theory of denaturation may be derived from analysis of the kinetic data. While the validity of the derivation of this theory may not be impugned, its application should be circumspect, for (1) the great majority of the data on protein denaturation do not merit quantitative interpretation, both because of the questionable purity of preparations and because of the inadequacy of experimental methods, and (2) the empirical values of the activation energy obtained from the Arrhenius equation may yet prove to be illusory in many cases.

An alternative method of interpretation of the abnormally large values of $\bar{E}$ is offered by Steinhardt (373) and La Mer (216). In view of the extraordinary pH dependence of reaction rate exhibited by protein denaturation (*cf. infra*), these authors suggest that part of the apparent energy of activation arises from

the heat of dissociation of the acidic equilibria involved in preparing the molecules for the kinetic step of activation. The customary method of calculating E by comparison of rates at constant pH is shown to be fallacious, inasmuch as the operation of the temperature coefficient of dissociation likewise effects a variation in the concentration of the charged species subject to the activation process. The advantage of this viewpoint is that it obviates the necessity of assuming large entropy changes of an uncertain nature. The method of appraisal has so far been restricted to pepsin (216, 373). An analogous interpretation for the acid denaturation of hemoglobin is offered in this review.

6. Application of the theory of absolute reaction rates to protein denaturation

To aid in an evaluation of the significance of the experimentally observed values of the activation energy, typical data for protein denaturation are given in table 7 and for enzyme inactivation in table 8. Since most of the existing kinetic data have already been amply reviewed (140, 372), we have selected those studies most amenable to quantitative interpretation both for reasons of purity of preparations and for explicitness of the kinetics. Without impugning the validity of the transition theory, one may well question the value of its application to impure systems. Similarly, while obedience to a first-order law is not implicit in the derivation of the theory, it does facilitate the computation of the thermodynamic functions and the comparison of rates, since the first-order constant is independent of concentration units. Moreover, by excluding reactions of indeterminate order one obviates questions as to the legitimacy of the use of unsteady constants.

In table 7 the kinetic data for the denaturation of hemoglobin are summarized. The conditions of denaturation have been taken from the original literature. In some instances, k' (given always for the lower temperature cited) is the value obtained by direct interpolation of the data for neighboring pHs. Where the rate exhibits a strong pH independence, the velocity constant read off from the graph of k' or $\log k'$ versus pH may differ significantly from the former value. This may lead to a value of E different in the case of pepsin, for example, by as much as 7000 calories. The values of the thermodynamic functions, $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta F^\ddagger$ are those given by Eyring and Stearn (140).

(a) *Hemoglobin*: The data may be summarized as follows: (1) In the neutral region, the rate varies markedly with pH, attaining a minimum at pH 6.76, but E decreases only insignificantly with pH, and $\Delta S^\ddagger$ and $\Delta F^\ddagger$ are essentially constant.

(2) At 6.76, the pH of minimum rate, the velocity constant at first increases to a maximum, then falls off with increasing salt concentration, while E rises continuously to a value of 120,000 calories, and $\Delta S^\ddagger$ nearly doubles. Ammonium sulfate displaces the minimum of the pH/ k' curve to the acid side. It should be noted that Lewis (228) reports that if calculation of E is made from the minima of the pH/ k' curves, or from the maxima of the ammonium sulfate/ k' curves, a steady value of about 79,000 calories is obtained. The former method may err in that comparison is not made at the same pH; the latter, in that it is not made

at the same salt concentration. However, in light of the view that rate comparison should be made at equal concentrations of the active species, there may be justification for this procedure.

(3) Even small amounts of alcohol (2 per cent and 4 per cent) effect a marked increase in the speed of denaturation. With higher concentrations the temperature of the experiment must be lowered, lest the rate become immeasurably fast. In analogy to the result observed with increasing salt concentration, the

TABLE 7
Kinetics of the denaturation of hemoglobin

SOLVENT	pH	T	k' (°)	E_{Ar}	$\Delta H^\ddagger$	$\Delta S^\ddagger$	$\Delta F^\ddagger$ (°)
		°C.	sec. ⁻¹	calories per mole	calories per mole	calories per degree-mole	calories per mole
Water (226)	5.7	60.5-68	4.3×10^{-4}	76,200	75,600	152.7	24,700
	6.8	60.5-68	1.05×10^{-4}	76,900	76,300	152.0	25,600
	8.0	60.5-68	3.35×10^{-4}	77,900	77,300	157.3	24,840
0.001 M ammonium sulfate (228).....	6.76	64-70	3.41×10^{-4}	77,300	76,500	152.5	25,100
1.14 M ammonium sulfate.....	6.76	64-70	5.45×10^{-4}	87,800	87,000	184.6	24,800
2.27 M ammonium sulfate.....	6.76	64-70	9.1×10^{-5}	104,600	103,800	230.9	26,000
3.03 M ammonium sulfate.....	6.76	64-70	2.44×10^{-5}	120,600	119,800	275.8	26,900
0 per cent alcohol (°) (68, 226).....	6.5	60.5-68	1.22×10^{-4}	75,750	75,050	148.5	25,550
20 per cent alcohol	6.5	40-45	3.3×10^{-5}	103,300	102,600	248.8	23,500
30 per cent alcohol	6.5	30-33	9.5×10^{-5}	120,800	120,100	319.5	23,300
Acid (121).....	4.85	37-45	8.0×10^{-5}	18,500	17,760	-19.9	23,900
	4.64	25-37	1.27×10^{-4}	12,100	11,300	-38.3	22,700
	4.52	18-25	1.42×10^{-4}	11,800	11,100	-37.8	22,100
	4.08	18-25	1.1×10^{-3}	11,700	11,100	-33.7	20,900

(°) k' given for the lower temperature listed.

(°) $\Delta F^\ddagger$ given for the lower temperature listed.

(°) Concentration expressed as volumes per cent.

value of E rises to a magnitude of 120,000 calories, and $\Delta S^\ddagger$ more than doubles. However alcohol, like ammonium sulfate, shifts the minimum of the pH/ k' curve to the acid side, and comparison at minimal rates instead of at constant pH would be of interest.

(4) The study of the denaturation of hemoglobin in the region of mild acidity (121) yields the surprising result that the energy of activation has undergone an apparent discontinuous drop to a more or less constant value of about 12,000 calories (pH 4.1-4.6). Correspondingly, $\Delta S^\ddagger$ has taken on a negative value. This reaction thereby assumes unusual interest, in that it is one of the rare in-

stances in protein denaturation for which the temperature coefficient is of the normal order of magnitude. It may be observed, moreover, that even in the acid region of constant E , the rate strongly depends upon pH.

(b) *Egg albumin*: It is of interest to note that in several respects the denaturation of egg albumin is consistent with that of hemoglobin: (a) At neutral reaction the rate reaches a minimum at pH 6.76, E being of the order of 130,000 calories (227). (b) The presence of concentrated salts depresses the rate (97); however, no initial maximum in rate is attained and E apparently drops (to 106,000 calories when calculated from second-order constants, or 88,000 if calculated from first-order "constants", for 2 M ammonium sulfate). (c) In the acid denaturation of egg albumin, E drops to about 97,000 calories at pH 3.4 (97) and to 48,000 at pH 2.3 (121), thereafter observing a small and rather continuous diminution to a constant value of about 36,000 over the pH range 1.1–1.7 (121). Moreover, in urea solution, the denaturation of egg albumin exhibits a negative temperature coefficient (192) corresponding to a heat of activation of about –6000 calories (140).

The meaning of the thermodynamic quantities $\Delta S^\ddagger$ and $\Delta F^\ddagger$ in terms of a theory of protein denaturation may now be examined. A cursory inspection of tables 7 and 8 will indicate that only a small variation obtains in $\Delta F^\ddagger$, or more precisely, in $\Delta F^\ddagger/T$. This is not surprising, for it is already evident from equation 4 that at constant temperature $\Delta F^\ddagger$ is the sole factor governing the rate of reaction. Accordingly, because of the logarithmic nature of the relationship, only a small variation in $\Delta F^\ddagger/T$ is admissible for the production of a conveniently measurable rate in the temperature region characteristic of protein denaturation.

Conversely, in the face of an extraordinary variation in $\Delta H^\ddagger$ under different conditions of denaturation, the maintenance of a rate of a given order of magnitude must be accompanied by a corresponding change in the entropy of activation. Thus, in table 7 a variation of 110,000 calories in $T\Delta S^\ddagger$ obtains for the denaturation of hemoglobin.

Just as the large entropy change of the over-all denaturation reaction (ΔS) is used as support for the suggested formation of random structures as a result of denaturation (278), the large *entropy of activation* ($\Delta S^\ddagger$) may be interpreted in terms of an "opening up" of the protein structure during the course of activation (140). While Mirsky and Pauling (278) have assumed that the molecule is held into its uniquely defined configuration by hydrogen bonds, Eyring and Stearn (140) have suggested that the specific configuration is maintained by the functioning of salt bridges as well as by bridges of the disulfide type. According to the latter theory, the energy required for thermal destruction of the cross linkages is compensated for by the large increase in entropy associated with the opening up of the specific configuration upon the breaking of the last few bridges.

In order, then, to distinguish between the degree of unfolding attributable to the *formation* of the activated complex and that associated with its "*flying apart*", it would be desirable to learn both ΔS and $\Delta S^\ddagger$. We have little information as to the former, and it is now profitable to consider what confidence may be attached to the values of $\Delta S^\ddagger$ given by the application of the theory of absolute

reaction rates to protein denaturation. Since $\Delta S^\ddagger$ is obtained by substitution of k' and $\Delta H^\ddagger$ in the rate equation, this is tantamount to a consideration of the validity of $\Delta H^\ddagger$, or more precisely of E , the experimental activation energy. This evaluation is also of interest in a consideration of the Mirsky and Pauling theory of protein denaturation, for in that variant, the number of hydrogen bonds broken is obtained by dividing E by 5000 calories per mole, the bond energy observed in simple systems.

7. Evaluation of the true activation energy

Steinhardt (373) and La Mer (216) have already pointed out that the time-honored method of the comparison of rates at constant pH may lead to adventitious values of E . This arises from the fact that acidic equilibria are involved in the preparation of the molecule for the kinetic step of activation. Thus



where P_0 is the initial protein molecule, P_n is the molecule after an n -fold ionization, and $P_n^\ddagger$ is the activated ionized complex. The operation of the temperature coefficient of dissociation in equation a promotes an increase in the concentration of P_n , the ionized species subject to activation. Thus, a comparison of rates at different temperatures and constant pH neglects the change in activity of the ionized molecules. This procedure leads to composite values of E ; for the heat of dissociation observed in equation a is added to the energy of activation involved in equation b.

True values of E may be obtained only by rate comparison at constant concentration of the ionized species, P_n (216, 373). The separation of the true energy of activation and the total heat of dissociation is most simply accomplished by a graphical method in which $\log k'$ is plotted against pH, the slope of this line corresponding to n . To the experimental points is fitted a theoretical curve corresponding to the equation

$$\log \frac{P_n}{P_0} = n(\log K_0 - \log a_H)$$

in which K_0 is the postulated dissociation constant. In this manner the apparent dissociation constant at a given temperature may be determined (K_0 being assumed identical for the n groups involved), and ΔpH corresponding to ΔpK may be calculated. Substitution in the equation

$$\Delta H = 2.3\Delta pH \cdot \frac{RT_2 T_1}{T_2 - T_1} \quad (5)$$

enables the calculation of the heat of dissociation of each group, this being multiplied n times to obtain the total ΔH . This value is then subtracted from the observed E , the remainder representing the true energy of activation.

(a) *Pepsin*: This method of appraisal has been applied by Steinhardt (373)

and La Mer (216) for the best-investigated case of protein denaturation, the alkaline inactivation of pepsin. Over a restricted pH range the rate was found to be inversely proportional to the fifth power of C_{H^+} . This pH dependence was ascribed to the dissociation of a proton from each of five primary amino groups of the initial molecule, leading to an increase of five in the net charge. This step corresponds to equation a, n being equal to 5. The quintuply ionized species was assumed to undergo the activation step in equation b.

Appraisal by the method already indicated yielded a value of 9040 calories per equivalent of protons dissociated, representing a total ΔH of 45,200 calories. The experimentally observed E was 63,500 calories, which upon correction for the heat of dissociation was reduced to a true value for the energy of activation of 18,300 calories.

The effect of this correction is to result in a change even in the order of magnitude of the apparent entropy of activation; at pH 5.7, for example, the corrected $\Delta S^\ddagger$ diminishes from 135.9 entropy units to 8.8 (*cf.* table 8). This small increase in entropy would indicate only an insignificant degree of unfolding of the activated complex. In terms of the Mirsky-Pauling theory of denaturation the correction in the value of E leads to a reduction of nine in the calculated number of hydrogen bonds broken, the corrected value of E corresponding to the destruction of but three to four hydrogen bonds.

While granting the validity of this general approach, Eyring and Stearn (140) contend that in this instance either the protein acts as a peculiar acid in the ionization process or the entropy change associated with the opening up of the protein molecule is disguised by the large entropy change of opposite sign involved in the ionization of the acid.

As Steinhardt (373) and La Mer (216) have pointed out, wherever the rate depends upon some power of the hydrogen- or hydroxyl-ion concentration, the existence of acidic equilibria may be suspected. In such cases the calculation of E by customary methods is fallacious.

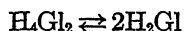
Moreover, the proponents of the absolute reaction rate theory have acknowledged (140) that "some, although by no means all, of the abnormally large activation entropies of protein reactions are due to failure to include the hydrogen-ion concentration properly in calculating the specific reaction rate constants". Inasmuch as this omission has been quite general in studies of the kinetics of protein denaturation, the calculation of the entropy of activation must be formal insofar as the value of the activation energy is empirical and the standard states of the reactants remain unspecified.

8. The acid denaturation of hemoglobin

A paucity of data has precluded the extension of Steinhardt and La Mer's method of appraisal to analogous reactions. However, we have recently observed another reaction in which the pH dependence of rate may be completely interpreted in a somewhat similar manner (333). This reaction is the acid denaturation of ox oxyhemoglobin over the pH interval 4.1-4.6, well investigated by Cubin in the temperature range 18-45°C. (121). A résumé of the phenomena accompanying hemoglobin denaturation follows.

The changes undergone in the thermal denaturation of oxyhemoglobin (60.5–68°C.), a reaction characterized by an apparent E of 77,000 calories over the pH range 5.7–8.0, are not known (226). From pH 5.7–4.85 a hiatus exists over which E apparently drops to 18,500 calories. From pH 4.6–4.1 a constant value of E of 11,800 calories is observed (121). Below this acidity the kinetics have not been studied.

The molecular-kinetic data are consistent with those of reaction kinetics. As will be discussed in a following section, the stability range of hemoglobin (pH 6–8) (377) corresponds to the region of thermal denaturation. There is evidence (*cf. infra*) to indicate that acid denaturation down to pH 4.1 causes the hemoglobin molecule to split into halves along the major axis, with no consequent unfolding of the molecule. The negative value of the entropy of activation, given in table 7, is in accord with the latter conclusion. The formation of acid hematin, accompanied by the breaking of the heme-globin bond, is not observed above pH 3.9, but is complete at pH 3.1 (190). It will be shown subsequently that the intactness of this bond inhibits the denaturation of the globin moiety, the latter reaction being characteristic of acid denaturation in 0.1 N hydrochloric acid. The partially reversible reaction occurring at room temperature in the restricted pH range 4.1–4.6 accordingly may be postulated as follows:



H representing heme, and Gl unaltered globin.

A plot of $\log k'$ against pH for the temperatures 18°, 25°, 37°, and 45°C. leads to a slope of exactly -2 (333), indicating that the rate is directly proportional to the square of the hydrogen-ion activity. Over the portion in which the curves are parallel and linear (pH 4.1–4.6), the heat of activation is constant and equals 11,800 calories. At higher pH, the curves diverge and E increases.

A fitting of the theoretical curve,

$$\log P_2/P_0 = -2(\log K_0 - \log a_H)$$

to the experimental points enables the evaluation of pK . At 25°C. pK equals 2.70, a value of the order observed for the carboxyl group of dipeptides (189). Similarly, the value of pK for other temperatures may be determined and the apparent value of ΔpK calculated. Substitution into equation 5 yields a heat of dissociation of 5600 calories per equivalent of protons, which, multiplied by 2, represents a total heat of dissociation of 11,200 calories. The subtraction of this quantity from the experimentally observed E yields a value of only 600 calories for the true Arrhenius heat of activation. Since in this temperature range RT equals about 600 calories, the true energy of activation, $\Delta H^\ddagger$, is zero. This conclusion is in accord with the suggestion (140) that if a "real" $\Delta H^\ddagger$ for the acid denaturation of hemoglobin is calculated, its value would not be greater than zero. The fact that $\Delta H^\ddagger$ is zero indicates either (1) that the rate of the reaction subsequent to ionization is independent of temperature, or (2) that the acid denaturation of hemoglobin results wholly from a twofold ionization.

In order to ascertain the mechanism of the denaturation it is incumbent to inquire about the meaning of a heat of dissociation of carboxyl groups of the order

of 5600 calories. Wyman (416) has shown that the apparent heat of dissociation of the carboxyl groups of horse oxyhemoglobin in the pK range of 4 to 5 is -2000 to -3000 calories. For amino acids, the values are of the order ± 1000 calories (110), leaving some 5000 calories to be accounted for by another process. In view of the fact that hydrogen bonding has been postulated as one of the principal mechanisms operating to maintain the intact configuration of the native molecule, and that the energy required for the thermal destruction of this bond is about 5000 calories (278), it is tempting to conclude that in each ionization process one hydrogen bond has been broken. Indeed, it is stated (278) that acids act to destroy hydrogen bonds by supplying protons individually to the electronegative atoms which would otherwise share protons (i.e., the oxygen atoms of the carboxyl group). In this process the small heat of ionization of the acid and that of hydrogen-bond disruption would be observed simultaneously, leading to an apparent value for the heat of ionization of the carboxyl groups of 5600 calories. This interpretation resolves the paradox associated with the observation of an appreciable temperature coefficient for the supposed pK , despite the well-known fact that the temperature coefficient of the ionization of carboxyl groups is negligible (110).

The conclusion reached by the present analysis is that the acid denaturation of hemoglobin in the pH range 4.1–4.6 proceeds by the addition of two protons and the breaking of two hydrogen bridges. It may be observed that the same conclusion may be attained more directly in the manner of Mirsky and Pauling (278), simply by ascribing the total energy of activation to hydrogen-bond disruption occasioned by individual protons supplied by the acidic environment. A dependence of the rate upon the square of the hydrogen-ion concentration, in addition to the fact that the heat of activation is approximately equivalent to twice the postulated bond energy, might be considered ample justification for the conclusion that two hydrogen bridges were broken in the activation process.

With regard to the structure of hemoglobin, it is of interest to observe that two is the least number of bonds that might be expected to maintain a rigid configuration for two subunits, and that if the halves are postulated to have the same structure, the number of bridges should be even.

The absence of kinetic data prevents an analysis of the changes taking place in the acid denaturation of hemoglobin below pH 4.1. Since the disruption of the heme-globin bond is initiated at pH 3.9, and denaturation of the globin may occur, the observed values of E may begin to change in this region just as they do above pH 4.6 with the onset of thermal denaturation. The fact should be noted that even in fairly strong acid (0.1 *N* hydrochloric acid) the formation of acid hematin and the denaturation of globin require some time (128).

Since the denaturation of egg albumin at room temperature in regions of greater acidity is consistent with that of hemoglobin, it may be predicted that in this other well-investigated case of protein denaturation, a similar pH dependence of the activation energy obtains. Indeed, it has already been shown that in the alkaline denaturation of crystalline egg albumin at 25°C. (87), the rate is proportional to $(OH^-)^4$. Likewise, in the thermal denaturation of to-

bacco mosaic virus, the reaction rate varies inversely with about the third power of the hydrogen-ion concentration (223), and in the urea denaturation of the same protein the rate depends on the reciprocal of about the 1.5th power (221). These results, together with the data for the acid denaturation (dissociation) of hemoglobin, substantiate the contention of La Mer—namely, that the abnormally large values of the activation energy of protein denaturation are illusory, and that if appropriate correction of them is made, it is unnecessary to invoke large entropy changes in an explanation of the observable rates.

TABLE 8
Heat inactivation of enzymes

PROTEIN	SOLVENT	pH	T °C.	k' sec. ⁻¹	E _{Ar} calories per mole	ΔH [‡] calories per mole	ΔS [‡] calories per degree- mole	ΔF [‡] calories per mole
Pancreatic lipase (251)	50 per cent glycerol	6.0	40-50	1.13×10^{-4}	46,000	45,360	68.2	23,960
Trypsin (311).....	24 per cent glycerol	6.51	50-60	2.83×10^{-5}	40,800	40,160	44.7	25,700
Enterokinase (312).....	Water	6.50	50-60	7.37×10^{-5}	42,800	42,160	52.8	25,100
Trypsinkinase (313)....	24 per cent glycerol	6.5	50-60	3.17×10^{-5}	44,900	44,260	57.6	26,200
Pancreatic proteinase (314).....	Water	6.9	50-60	1.27×10^{-4}	38,500	37,860	40.6	24,700
<i>Alkaline inactivation of pepsin (140, 373)</i>								
<i>Process</i>								
$P_0 \rightarrow P_s^{\dagger}$		6.44	15-25	1.89×10^{-5}	56,300	55,600	113.3	23,000
$P_0 \rightarrow P_s^{\ddagger}$		5.7	15-25			67,470	135.9	
$P_s \rightarrow P_s^{\ddagger}$		5.7	15-25			22,360	8.8	
$P_0 \rightarrow P_s$		5.7	15-25			45,110	127.1	

9. Biological inactivation

Table 8 gives the kinetic and thermodynamic data for the heat inactivation of a number of well-investigated enzymes, as well as for the alkaline inactivation of pepsin. With the exception of pepsin, the preparations were non-crystalline but were purified by the methods of Willstätter and Waldschmidt-Leitz. In several cases the purity is questionable: for example, the product called "trypsin" (311) has since been acknowledged to contain two fractions, one being pancreatic proteinase, an enzyme analogous to the classical trypsinogen, and the other a carboxypolypeptidase (314). Similarly, enterokinase (studied with the presence of trypsin (312)) is included as an "enzyme" to facilitate comparison with trypsinkinase, the product obtained upon the interaction of "trypsin" with its specific activator, enterokinase (313).

The heat inactivation of the enzymes in the upper section of table 8 is characterized by about the same value of E . Indeed, the constancy of E for the

four similar proteinase preparations evokes suspicion that the same inactivation process may have been measured in each instance. It should be noted that the concentration of glycerol is without effect on the E values for purified enzymes (251, 314). However, the purity of the enzyme is unquestionably important, for McGillivray (251) found that the E of unpurified lipase dropped from the order of 100,000 calories to 35,000 calories with changing glycerol concentration.

In all these cases a marked variation of rate with pH was noted, the plot of the pH/k' curve reaching a minimum at pH 6.0 for pancreatic lipase, and at pH 6.5 for the proteinases. (The similarity in the pH of minimal rate in the case of the proteinases is another indication that the same function was being measured in all instances.) These minima are to be compared with those already given for hemoglobin and egg albumin, i.e., pH 6.76 in both instances. The existence of such minima in the region of neutrality (about 6.76 at 37°C.) has led to the suggestion that both hydrogen and hydroxyl ions are involved in the catalysis of denaturation (226). The fact that hydrogen ions are indeed involved in the dissociation of hemoglobin at pH 4.1–4.6 has already been discussed. This strong dependence of rate on pH is further evidence for the contention that the abnormal values of E may be adventitious.

Moelwyn-Hughes (281) has ably reviewed the mechanism of the inactivation of these enzymes on the basis of the collision theory of reaction kinetics.

10. Variation of the activation energy with solvent, pH, and temperature

The previous discussion has been concerned principally with the kinetics and thermodynamics of *thermal* denaturation and the explanation of the paradox associated with the presence of abnormally large activation energies. It has already been observed that the activation energy may differ greatly with the conditions of denaturation. For example, in table 7 the activation energy for the denaturation of hemoglobin is shown to vary over a whole order of magnitude, changing from 11,800 calories at pH 4.1–4.6 to 120,000 calories for the thermal denaturation in the presence of 30 per cent alcohol or 3 *M* ammonium sulfate. To attribute the success of each of these processes to the selfsame reaction step would be absurd. It is more profitable to attempt to break the reaction mechanism down into several successive steps, a procedure that seems admissible for the case of hemoglobin at least.

It is not feasible to list in order the result of different environmental conditions upon E ; for, whereas increasing the concentration of ammonium sulfate effects a consistent increase in E for hemoglobin (228), an apparent decrease is observed for egg albumin (140). However, it appears to be a valid generalization that in acid solution the activation energy tends to a minimum value, and that in urea solution a change in sign may even be observed.

It has already been noted that in the denaturation of both egg albumin and hemoglobin, the activation energy assumes a constant minimum value at low pH. The same effect is observed in the destruction of ptyalin, for which E drops from about 123,000 calories at pH 6.8 and higher to 38,500 calories at pH 3.2 (134). Furthermore, the rate of the acid denaturation of edestin is the same at 2°C.

as at 20°C., that is, the temperature coefficient is unity and the activation energy is zero (39).

In terms of the Steinhardt-La Mer concept of the importance of ionization processes in denaturation, the effect of acidity upon E is not surprising, for in acid solutions only carboxyl groups may be dissociating, and the effect of temperature upon their dissociation is negligible (373). Correspondingly, the large temperature effect experienced in alkaline solution may in part be attributed to the ionization of amino or other basic groups whose temperature coefficient is appreciable. An alternative explanation may be found in the suggestion (140) that salt bridges may be broken either thermally or by neutralization due to a pH shift. In the latter event the heat of neutralization involved will not be observed in the measurement of the activation energy. Thus, if the breaking of salt bridges is assumed to be an integral part of the denaturation mechanism, the activation energy may decrease with pH.

Contrary to the behavior experienced for most chemical reactions and especially in contrast to that observed in the thermal denaturation of proteins, the rate of urea denaturation of egg albumin exhibits a negative temperature coefficient (192, 334), corresponding to an activation energy of about -7000 calories (140). Even more remarkable is the urea denaturation of tobacco mosaic virus (221, 371). In this reaction the rate observes a minimum at room temperature and is governed by a negative temperature coefficient below room temperature and a positive coefficient above. Other viruses exhibit similar anomalies (52). On the other hand, a positive coefficient appears to hold for the urea denaturation of fibrinogen (127) and carboxyhemoglobin (128).

The unusual temperature-rate effect observed for tobacco mosaic virus may be explained by the assumption that the native nucleoprotein is transformed into the denatured material by two (or more) simultaneous reactions, the one governed by a positive temperature coefficient, the other by a negative coefficient, the over-all effect being the resultant (221). If the two parallel processes are supposed to differ only in the number of urea molecules combining with a given virus particle, it may be predicted that the temperature coefficient would vary not only with temperature but also with urea concentration, for both these factors would be expected to govern the extent of combination.

The effect of urea upon the kinetics of protein denaturation has been ascribed to the entropy change associated with a "freezing out" of urea as the result of the strong bonding of the urea to the activated complex (140).

In concluding the discussion of the activation energy, it should be pointed out that the universally assumed temperature independence of the activation energy is not always observed in protein denaturation even over the limited temperature range available for study. A number of examples are known for which the activation energy varies markedly with temperature (e.g., ptyalin (134)) or where the plot of the $\ln k'$ versus $1/T$ deviates from linearity (407). Sufficient data are not available for the evaluation of the significance of this anomaly. However, an interesting analogy is to be found in the variation with temperature of the critical increment of *flocculation* for denatured horse serum. Lewis's

recalculation (230) of the data of Chick and Martin (99) shows that E decreases from 110,000 calories per mole at 44°C. to a constant value of about 23,000 calories at 76–86°C. Steinhardt has observed (373) that the fact that E becomes smaller and approaches a limiting value at higher temperatures is in accord with the effect of temperature upon the dissociation of ionizable groups, a process supposed to precede the activation step.

11. The heat of reaction

The heat of reaction, ΔH , is equal to the difference between E_1 , the energy of activation of the forward reaction, and E_2 , the energy of activation of the reverse reaction. E_1 may be obtained by kinetic studies, and $\Delta S_1^\ddagger$, the entropy of activation, may be evaluated by the Eyring method. The heat of reaction or heat of denaturation, ΔH , is measured thermochemically or by substitution in the van't Hoff equation expressing the variation in the equilibrium constant with temperature. From the well-known relations between free energy and the equilibrium constant, and between free energy and the heat of reaction, the entropy change ΔS may be obtained. If the denaturation process is reversible, from the above information one may then calculate E_2 , and $\Delta S_2^\ddagger$ for the reverse reaction.

A knowledge of the heat change and the entropy change involved in both the activation step and the over-all reaction is fundamental for the interpretation of the mechanism of denaturation. It is therefore evident that a combination of thermochemical and kinetic data would aid greatly in the elucidation of the denaturation process. Unfortunately, both kinetic and thermochemical measurements have not yet been made upon the reversal of the denaturation process for any protein under the same conditions. The one instance where somewhat comparable data are available is the equilibrium governing the "reversible" acid denaturation of trypsin, as studied by Anson and Mirsky (17). In 0.01 *N* hydrochloric acid (pH 2–3) the heat of reaction calculated from the variation of the equilibrium constant with temperature is 67,600 calories per mole (17), and evaluation of the entropy change leads to a value for ΔS of 213 entropy units (372). The kinetic data of Pace (311) are for crude preparations of trypsin subjected to heat inactivation at neutral pH. The heat of activation of denaturation (*cf.* table 8) is less than 41,000 calories and the entropy of activation is but 44.7 units. If the legitimacy of the extrapolation of Pace's data to the acid region is tentatively assumed, the anomalous situation arises that the slow reverse reaction has a *negative* heat of activation of 27,000 calories per mole. However, from the above data the entropy of activation of the reverse reaction is seen to be –168.4 units per mole. Stearn (372) has suggested that it is this large loss of entropy which results in the slowing up of the reverse reaction.⁶

⁶ The magnitude of the heat change, entropy change, and activation energy of denaturation of trypsin cited by Mirsky and Pauling (278) and apparently derived from the data of Northrop (305) differs considerably from the figures given above. Northrop (305) has stated that his experiments were not sufficiently accurate to permit calculation of the exact temperature coefficient. On the other hand, the extrapolation of Pace's data appears questionable. It is evident that the calculation of the number of hydrogen bonds broken in the denaturation process or of the associated entropy change still remains qualitative.

The heat of reaction of a number of other denaturation processes has been determined calorimetrically and further application of the method seems desirable. The irreversible denaturation of methemoglobin at pH 9–12 (25°C.) has been studied by an indirect calorimetric procedure, both at constant quantity of alkali in the solution and at constant pH (117). The heat of denaturation of the protein by alkali is obtained from the difference in the heats of reaction of native and denatured protein with potassium hydroxide. As a result of a pH change during denaturation the heat change at constant pH is less than that at constant quantity of alkali. At constant alkali the heat of denaturation of methemoglobin is reported to be 138 (± 14) kcal. per mole, about twice that reported by Anson and Mirsky (17) for trypsin, and at constant pH is about 100 kcal. In this study, the important conclusion was reached that, contrary to statements made in the literature (5), denaturation is not an "all or none" process and the insolubility of methemoglobin at the isoelectric point is not a good measure of denaturation.

A similar study on the heats of reaction of methemoglobin and carboxyhemoglobin with sodium salicylate at pH 7.3 yielded no difference in the heats for either protein at a given salicylate concentration (336). Moreover, the heat of reaction of methemoglobin increased with salicylate concentration up to 0.8 *M*, leading to the conclusion that the reaction was not stoichiometric and that part of the heat of reaction probably arose from hydrogen-bond formation between the salicylate and the protein. This result, together with others reported in Part IV, indicates the need for a modification in the theory of the existence of a mobile equilibrium between native and denatured protein (18) insofar as this is based upon the reaction between hemoglobin and sodium salicylate.

In view of the extensive kinetic investigation of the irreversible alkaline inactivation of crystalline pepsin (373) and of the importance attached to its interpretation, the calorimetric measurement of the heat of denaturation of crystalline pepsin by alkali is significant. The surprising result was obtained (116) that the heat of denaturation fell off with pH from 85 kcal. per mole of pepsin at pH 4.3 to a negligible value at pH 6.8.⁷ From pH 4.3 to 6.2, ΔH decreased slowly, but thereafter it decreased abruptly. On the other hand, the enzymatic activity remained constant to pH 6.2, thereafter falling off 70 per cent in going to pH 6.8. That ΔH and enzymatic activity are different functions of pH emphasizes the fact that the measured heat of denaturation must be regarded as the sum of changes in heat content due to several chemical processes, of which enzymatic inactivation is but one, and that these may not as yet be distinguished. This view is in accord with that already expressed in this review—namely, that denaturation consists of a series of related changes, of which biological inactivation is but one aspect.

In analogy to Steinhardt's kinetic results (373) it is interesting to observe that at pH 6.2 and 30.6°C. (the temperature of the calorimetric experiment)

⁷ The heat of denaturation of crystalline *pepsinogen* calculated from the effect of temperature on the equilibrium between the native and denatured forms at pH 7.0 is 31 kcal. per mole (185).

the amount of pepsin in the ionized form is insignificant, whereas at pH 6.8 ionization is virtually complete and the energy of activation apparently undergoes a change in value. Since the kinetic experiments were made in buffered solutions at constant pH, and the calorimetric measurements were undertaken at varying initial pH in the presence of different amounts of alkali, and since the reaction has been shown to be essentially irreversible, the data are not amenable to a comparison in the manner given for trypsin.

III. PROPERTIES OF DENATURED PROTEINS

A. MOLECULAR WEIGHT

Molecular-kinetic properties of proteins can be described in terms of size and shape. Information as to the change in these properties upon denaturation is of vital importance for the characterization of denatured proteins. Measurements of (a) osmotic pressure, (b) sedimentation and diffusion, and (c) diffusion and viscosity have been used to determine the molecular weight of denatured proteins. On the basis of these measurements, proteins may be divided into three groups: (1) those which have the same molecular weight in the denatured as in the native state; (2) those which are split; and (3) those which become aggregated upon denaturation. In the following sections the experimental data are presented and discussed according to this classification.

1. Denaturation causing no change in molecular weight

In table 9 data are given relative to the nature of the protein and of the denaturing agent, the molecular weight, and the method used for its determination. Within the limits of the resolving power of the methods, the molecular weight of the denatured protein is identical with that of the parent native material.

(a) *Egg albumin*: Comparative osmotic-pressure measurements yielded a value of 35,000 for the native protein in buffered solutions of pH 4.8 (36), and of 36,000 (36) and 32,000 (193) in 6.66 M^* urea solution. Although the molecular weight of the native protein has since been found to be somewhat higher, i.e., about 45,000 (77, 381), it seems to be established that urea denaturation does not affect the molecular weight of egg albumin. While Williams and Watson (406) believed that the observed decrease in the sedimentation constant of the protein upon treatment with 6.66 M urea was due to a splitting of the molecules into halves, viscosity data indicate that the decrease in sedimentation rate is due to a change not in molecular weight but in molecular asymmetry (75). This is in accord with more recent sedimentation and diffusion measurements (347). Denaturation of egg albumin by acid alcohol or alkaline alcohol, followed by denaturation by urea, likewise yields a protein of essentially the same molecular weight as the native form (193). However, if the protein is first denatured by acids or alkali, changes in mean molecular weight occur (193).

(b) *Serum albumin (horse)*: Osmotic-pressure measurements of Burk (80)

* In this and the following sections M denotes molar concentration.

TABLE 9
Proteins which undergo no change in molecular weight upon denaturation

PROTEIN	DENATURING AGENT	METHOD OF MEASUREMENT	MOLECULAR WEIGHT		REFERENCE
			Native	Denatured	
Egg albumin . . .	6.66 <i>M</i> urea	Osmotic pressure	35,000	36,000	(36)
	6.66 <i>M</i> urea	Osmotic pressure		32,500	(193)
	6.66 <i>M</i> urea	Sedimentation,		32,000	(75, 406)
		diffusion- viscosity	44,000	44,000	(347)
Serum albumin (horse).	6.66 <i>M</i> urea	Osmotic pressure	74,600	73,800	(80)
	8 <i>M</i> urea	Diffusion-vis- cosity	71,900	77,800	(295)
	8 <i>M</i> guanidine hydrochloride	Diffusion-vis- cosity		80,600	(295)
	Heat and 6.66 <i>M</i> urea	Osmotic pressure		72,900	(80)
Serum globulin (horse).....	6.66 <i>M</i> urea	Osmotic pressure	175,000	173,000-	(3, 82)
		Sedimentation- diffusion	167,000	178,000	(cf. 381, p. 374)
	8 <i>M</i> urea	Diffusion-vis- cosity	170,000	170,000	(296)
Hemoglobin and globin (sheep and dog).....	6.66 <i>M</i> urea	Osmotic pressure	65,300- 66,600	63,000- 69,400	(415)
Gliadin.....	6.66 <i>M</i> urea	Osmotic pressure	40,900	44,200	(83)
	1.7 <i>M</i> urethan	Osmotic pressure		42,000	(83)
Zein.....	8.33 <i>M</i> urea	Sedimentation- diffusion	40,000	40,000	(399)
Pepsin.....	1 <i>M</i> urea, 4 <i>M</i> urea, 6.5 <i>M</i> acetamide	Sedimentation	$S^* = 3.0$	$S = 3.3$	(374)
Tomato bushy stunt virus.....	Heat, freezing	Sedimentation	$S = 133$	$S = 133$	(308)
Thyroglobulin ...	Heat in salt- free solu- tions, urea, amino acids, high protein concentra- tion	Sedimentation	$S = 17.8$ 650,000	$S^\dagger = 10$ 650,000	(245, 249)

* S = sedimentation constant.

† Interpreted as being due to change in molecular shape (245).

yielded the same molecular weight for native and for urea-denatured horse serum albumin. Combined diffusion and viscosity measurements revealed variations in apparent molecular weight following denaturation by increasing concentrations of urea (300). These have since been traced to the limitations of Kuhn's equation for the interpretation of viscosity data, as well as to an apparent retardation of the diffusion rate in concentrated urea solutions (295). Later work, on a crystalline fraction of horse serum albumin (295), indicated the molecular weight of the protein, denatured by 8 *M* urea or 8 *M* guanidine hydrochloride, to be essentially the same as that of the native form. This has been found to hold also for bovine serum albumin (331).

Although serum albumin heated at 70°C., and dissolved in 6.66 *M* urea, has the same molecular weight as the native protein (80), it appears that in this instance urea caused a depolymerization of the aggregates of heat-denatured protein (118). Sodium salicylate, in concentrations of 1 *M* and 3.5 *M*, is without effect on molecular size, as revealed by diffusion data (300).

(c) *Serum globulin*: Recent estimates of the molecular weight of crude preparations of native horse serum globulin vary from 150,000 (337) to 175,000 (3, 82; cf. also 381, p. 354 ff.). In 6.66 *M* urea, Burk (82) observed a value of 173,000 to 178,000. Combined diffusion and viscosity data also indicate no change in molecular weight of an electrophoretically monodisperse preparation of pseudoglobulin upon denaturation by concentrated urea solutions (296).

(d) *Hemoglobin (sheep and dog)*: Unlike those of the horse, beef, and ox, the methemoglobins, oxyhemoglobins, and globins of sheep and dogs are not split by concentrated urea solutions (415).

(e) *Gliadin*: This protein, although not homogeneous in the ultracentrifuge (207), was found by Burk (83) to have about the same mean molecular weight in 50 to 60 per cent ethanol (40,000) as in 6.66 *M* urea solutions. Progressive aggregation occurs, however, when the buffer components of the urea solutions exceed a concentration of about 0.1 *M* phosphate. Urethan which, like urea, liberates free disulfide groups, has, in the absence of buffer components, no effect on the molecular weight of the protein.

(f) *Zein*: Comparative ultracentrifugal analysis in 45–49 per cent ethanol, and in 50 per cent urea solutions, failed to reveal any difference in sedimentation rate when the protein was exposed to urea for about 5 hr. (*S* = 1.9; *M* = 40,000). After prolonged contact with urea, fragmentation occurred (399).

(g) *Pepsin*: Exposure of crystalline pepsin to 1 and 4 *M* urea solutions or to 6.5 *M* acetamide for 1 to 3 hr. produces only a small increase in the sedimentation constant of the native protein (374). While diffusion measurements have not been recorded, it appears that interaction between pepsin and these amides does not involve a change in molecular weight. Enzymatic activity also was unimpaired by 3 hr. exposure to 4 *M* urea. Yet it remains to be seen whether prolonged exposure to these or higher concentrations of these amides does not exert appreciable effects on the particular properties studied.

(h) *Tomato bushy stunt virus*: Inactivation of this virus protein by heating or by freezing does not alter its sedimentation rate (308).

(i) *Thyroglobulin*: The sedimentation behavior of thyroglobulin is changed

either by raising the protein concentration of salt-free solutions or by adding urea, glycine, tyrosine, and related compounds, even in the presence of electrolytes (245, 249). The decrease in sedimentation constant, from $S = 17.8$ to $S = 10$, has been interpreted as being due to an unfolding of the molecules without any concomitant changes in size (α -protein). Although the original sedimentation constant becomes restored upon reversing the processes applied for the formation of α -protein, it is not known whether the reconstituted protein answers all the criteria of the native material. The α -protein is readily converted irreversibly into a more highly denatured form, or reversibly into fragments of lower molecular weight. Similar findings have been obtained with antitoxic diphtheria pseudoglobulin and with thymus nucleohistone. Further investigation of these processes by means of diffusion or viscosity measurements is needed before the interpretation of the ultracentrifugal measurements can be fully accepted.

2. Denaturation causing splitting of protein molecules

Molecular dissociation and denaturation

Denaturation of a number of proteins has been found to be accompanied by splitting of the molecules into subunits of well-defined kinetic properties. Whether splitting is an accompaniment of denaturation depends on the internal configuration of the protein molecule. Such proteins as the hemocyanins (72) or phycocyan (138) are capable of undergoing molecular dissociation under conditions which ordinarily are considered too mild to cause denaturation (as revealed by the liberation of sulfhydryl groups or other denaturation criteria). Edestin, excelsin, the hemoglobins and other proteins listed in table 10 are readily dissociated by typical denaturing agents, whereas serum globulin (296) and myogen (154) are so after, or concomitant to, unfolding of the molecule. Other proteins, i.e., those considered in the preceding section, resist dissociation even in the denatured state. This divergence in susceptibility to molecular fission may be related to the physical configuration of the protein molecules, in that some proteins are composed of structural subunits into which they are readily separated, whereas others consist either of a single structural unit or else of subunits which do not separate even when the protein is denatured (289).

Upon moderate variations of pH, or upon the addition of electrolytes, *Helix pomatia* hemocyanin dissociates into subunits having one-half and one-eighth of the molecular weight of the native protein (72). The dissociated molecules appear to have the same molecular shape as they have when combined in the whole molecule, as indicated by calculations of axial ratios (*vide infra*). The molecule splits lengthwise or across (289). The subunits recombine upon reversing the conditions applied for dissociation. When myogen is acted upon by urea it dissociates into halves; however, the subunits undergo further changes in that they unfold as they dissociate (154). The same is true of horse serum globulin when treated with 3 *M* guanidine hydrochloride (296). Obviously these two types of reactions are of different magnitude.

The molecular weight corresponds to the smallest molecular-kinetic unit which still reflects all chemical, physical, and biological properties of the protein

in bulk. If the subunits of hemocyanin or hemoglobin deviate in any single property from the whole molecules,—as they do, since they carry only one-half of the complement of prosthetic groups,—dissociation is to be considered as one type of denaturation. If a more fundamental change in properties is accepted as a criterion for denaturation, such as an intramolecular rearrangement, the dissociation of these proteins is a process different from that occurring when myogen is dissociated. Similar considerations apply for the process of recombination of the subunits. If the recombined hemoglobin or hemocyanin is identical in all respects with the original native protein, dissociation is truly reversible; otherwise it is not. However, the extent to which the original properties can be regained depends on the extent of change occurring upon dissociation: dissociation without alterations in the configuration of the subunits is more nearly reversible than dissociation with simultaneous intramolecular changes.

It was found that although the recombined molecules of *Helix pomatia* hemocyanin and of hemoglobin had the same size and shape as those of the parent native proteins, slight differences were observed in the electrophoretic mobility for hemocyanin (393) and in the shape of the oxygen dissociation curve, as well as in resistance to splitting by alkali, for hemoglobin (see below). That the method applied for dissociation may have a profound influence on the extent of recombination may be seen from the fact that with *Helix pomatia* hemocyanin recombination occurred fully when the protein was dissociated by pH changes or salts, partly when it was dissociated by ultraviolet light, and not at all when it was dissociated by ultrasonic waves (72).

An explanation for these observations may be found by considering the variations in the degree of denaturation, as exemplified in the introduction by the A-Z scale. Assume that the dissociation of hemocyanin by salts represents a change in properties corresponding in extent to a change from state A (native) to state G; that of hemoglobin by acids, where —SH groups become detectable (276), from A to L; and that of myogen by urea (154), where the subunits unfold, from A to P. All three types of dissociation are denaturation reactions in the broadest sense; however, the most apparent change in the first instance is molecular dissociation; in the latter instances, it is dissociation accompanied by further intramolecular changes.

It may be seen, therefore, that mere dissociation of protein molecules into subunits is no indication of the degree to which denaturation occurred, and that a change in molecular weight may be the result of processes of quite different orders of magnitude. An evaluation of the latter requires the application of additional standards of reference, both for the dissociation reaction and for its reversal. This will be borne out by a more detailed discussion of the dissociation of specific proteins, presented in the following paragraphs.

Experimental results

In table 10 the proteins are listed which undergo dissociation and denaturation.

TABLE 10
Proteins which undergo dissociation upon denaturation

PROTEIN	DENATURING AGENT	METHOD OF MEASUREMENT	MOLECULAR WEIGHT		REFERENCE
			Native	Denatured	
Myogen.....	6 M urea	Sedimentation-diffusion	150,000	72,000	(154)
	6.66 M urea	Osmotic pressure	(81,000)	(34,000)	(401)
Serum globulin (horse).....	3 M guanidine hydrochloride	Diffusion-viscosity	170,000	96,000	(296)
Egg albumin	N/20 sodium hydroxide	Osmotic pressure	36,000	16,000	(193)
Edestin.....	6.66 Urea	Osmotic pressure	310,000	49,500	(36)
	Hydrochloric acid	Osmotic pressure		17,000	(2)
	Hydrochloric acid	Sedimentation	$S^* = 12.8$	$S = 2.3$	(39)
Excelsin.....	6.66 M urea	Osmotic pressure	214,000	35,700	(81)
Amandin	6.66 M urea	Osmotic pressure	206,000	30,300	(81)
Casein.....	6.66 M urea	Osmotic pressure	75,000-100,000	33,000	(36)
<i>Limulus</i> hemo-hemocyanin ..	6.66 M urea	Osmotic pressure	2,040,000	142,000	(84)
	Hydrochloric acid followed by 6.66 M urea	Osmotic pressure		69,000	(84)
Hemoglobin (horse, beef) ..	Hydrochloric acid	Sedimentation-diffusion	69,000	39,000	(155)
		Osmotic pressure		39,000	(343)
	Urea	Osmotic pressure	69,000	35,800	(193)
	Urea, formamide, acetamide	Sedimentation-diffusion		39,000	(374)
Tobacco mosaic virus.....	6 M urea	Osmotic pressure	40×10^6	40,000-100,000	(371)
	Pressure	Sedimentation		See text	(222)
Myosin.....	Urea	Osmotic pressure	1,000,000	100,000	(409)

* S =Sedimentation constant.

TABLE 10—*Concluded*

PROTEIN	DENATURING AGENT	METHOD OF MEASUREMENT	MOLECULAR WEIGHT		REFERENCE
			Native	Denatured	
Pepsin.....	Alkali	Sedimentation	40,000	See text	(323)
Diphtheria antitoxic globulin.....	Pepsin	Sedimentation-diffusion	184,000	98,000	(322, 391)
Insulin.....	Duponol	Sedimentation-diffusion	41,000	27,500 See text	(262)
<i>Helix pomatia</i> hemocyanin...	pH, salts, non-electrolytes, ultrasonics, ultraviolet light, and α -rays	Sedimentation-diffusion	8.9×10^5	4.31×10^5 1.03×10^5	(72)

(a) *Myogen*: Earlier osmotic-pressure measurements by Weber and Stoeve (401) yielded a molecular weight of 81,000 for the native protein and of 34,000 for the protein denatured by 6.66 *M* urea. These data have been superseded by the more accurate sedimentation and diffusion measurements (154), yielding values of 150,000 and 72,000, respectively. As will be discussed in greater detail in Part III B, the denatured half-molecules are considerably more asymmetric than would be expected to result from mere dissociation.

(b) *Serum globulin (horse)*: Whereas denaturation by 8 *M* urea is without effect on the molecular weight of horse pseudoglobulin (296), profound changes occur upon denaturation by guanidine hydrochloride. In 2 *M* solutions of this salt aggregation occurs, while in concentrations higher than 3 *M* the protein molecules split into halves as they unfold (296). Under these conditions, horse serum globulin follows the pattern of the urea denaturation of myogen.

(c) *Egg albumin*: Treatment of egg albumin with *N*/1 sodium hydroxide gives rise to a polydisperse material of allegedly greatly reduced antigenic activity (384). Huang and Wu (193) obtained an osmotic molecular weight of $15,900 \pm 3,600$ when egg albumin was exposed to *N*/20 sodium hydroxide for 24 hr., and when the precipitate, obtained after neutralization, was dissolved in 40 and 50 per cent urea. The liberation of ammonia and hydrogen sulfide, noted upon neutralization, indicates that more far-reaching, chemical changes had occurred.

(d) *Edestin*: Denaturation by 6.66 *M* urea reduces the molecular weight from 310,000 (36) to 49,500. At the same time four-ninths of the total sulfhydryl groups become exposed (161). Though about the same amount is set free upon denaturation by hydrochloric acid (39), splitting proceeds farther, resulting in the formation of subunits of molecular weight about 17,000 (2). The latter

reaction has been the subject of a careful study by Bailey (39), who has used several physical and chemical methods to compare the properties of the native, denatured, and reconstituted protein.

(e) *Excelsin*: Only a small fraction of the total sulfhydryl groups is liberated upon denaturation by 6.66 *M* urea. At the same time, the protein splits into subunits of one-sixth the molecular weight of the native material (35,700 as compared to 214,000). Dilution of the urea solutions with water results in complete precipitation of the denatured protein (81).

(f) *Amandin*: This protein, as previously noted, is devoid of cysteine residues. Treatment with 6.66 *M* urea lowers the molecular weight from 206,000 to 30,300 (81).

(g) *Casein*: This protein is polydisperse in the ultracentrifuge, with a main component having a molecular weight of 75,000–100,000 (381). Treatment with 6.66 *M* urea yields an osmotic molecular weight of 33,000 (36).

(h) *Limulus hemocyanin*: This copper-containing respiratory blood protein of the horseshoe crab has a molecular weight of 2,040,000 (381). Treatment with 6.6 *M* urea causes decolorization of the protein, renders it insoluble in isoelectric salt solutions, and lowers the osmotic molecular weight to 142,000 (84). The dissociated molecules contain one prosthetic group with four atoms of copper. If hemocyanin is first denatured by acids, which split off copper, and then by 6.66 *M* urea, further dissociation occurs, yielding a molecular weight of about 69,000, which is about twice the minimum molecular weight as determined by chemical analysis. *Cancer hemocyanin* (208) behaves differently in that the native and the copper-free protein are dissociated to the same extent by concentrated urea solutions. It appears, therefore, that in *Limulus hemocyanin* the copper is combined in such a way that a breakage of its linkages leads to further dissociation (84).

(i) *Hemoglobin and its derivatives*: We have mentioned before that the hemoglobins of the horse, beef, and ox, unlike those of the sheep and dog, are dissociated by acids and by urea and other amides. Since the literature on this subject is widely scattered, the dissociation of hemoglobin and globin will be discussed here in some detail.

(1) *Dissociation by acids*.—When horse or beef hemoglobin is treated with 0.1 *N* hydrochloric acid, it dissociates into hemin and globin (11, 190). The latter can be precipitated by acetone, dried, and redissolved in water. This acid-denatured globin is somewhat heterogeneous in the ultracentrifuge (155), with a mean sedimentation constant of about $S = 2.5$. The same value is obtained for acid-treated globin prior to the precipitation by acetone. It reveals a certain proportion of sulfhydryl groups under conditions at which the native hemoglobin reveals none (276). Rapid neutralization of acid-denatured globin yields a water-insoluble precipitate which, when dissolved in alkali, combines with heme to form hemochromogen, a pigment incapable of combining reversibly with oxygen (11). This differs from native hemoglobin in absorption spectrum. Careful neutralization of acid globin, at low temperatures, yields a fraction of a material which is soluble under conditions at which denatured

globin is not. This allegedly "native" globin (11) is now devoid of free —SH groups (276) and, although also somewhat heterogeneous, gives a sedimentation constant of $S = 2.5$ and a diffusion constant of 6.5×10^{-7} , equivalent to a molecular weight of 39,000 (155), which is in close agreement with the value derived from osmotic-pressure measurements (343).⁹ It can be recombined with heme to form a substance identical with native hemoglobin in absorption spectrum (11, 190, 191) and molecular size and shape ($M = 69,000$ (155, 342)); however, it is somewhat less homogeneous, more susceptible to dissociation by alkali (beef (339)), and differs also in electrophoretic mobility (155) and in the shape of the oxygen dissociation curve (187). The present evidence indicates that acid treatment of hemoglobin causes not only dissociation of the molecule into halves, but also further denaturation of the dissociated subunits. What has been referred to as "native" globin is actually a regenerated product, obtained after neutralization of a solution of the denatured protein. The mere fact that it is as soluble as the native globin, is devoid of free sulfhydryl groups, and is capable of recombination with heme so as to form a substance resembling native hemoglobin is not unequivocal proof for its genuine character. Its preparation from a denatured material, together with the established differences in stability (339) and other physicochemical and biological properties already noted, suggests strongly that it is different from the globin moiety of native hemoglobin (Part IV).

(2) Dissociation by urea and related amides.—Treatment of horse (36) and ox hemoglobin (193) with concentrated urea solutions likewise causes dissociation into half-molecules, as determined by osmotic-pressure measurements. The effects of varying concentrations of urea, acetamide, and formamide on horse hemoglobin have been studied extensively by Steinhardt by means of sedimentation, diffusion, and other physical measurements (374). The sedimentation constant of native carbon monoxide hemoglobin was reduced from $S = 4.63$ to 3.2 by 4–7.5 M urea solutions, to 4.06 by 3 M acetamide, and to about 3.5 by 4.5–6.5 M solutions of acetamide. 4.55 M formamide reduced the sedimentation constant to $S = 3.0$, while 2 M glycine was without effect. These particular changes were complete about 1 hr. after the addition of the dissociating agents and resulted in a molecular weight of about 39,000. Unlike hydrochloric acid, these amides did not cause the formation of denatured globin, or even the splitting of the heme from the globin moiety. There was no appearance of protein sulfhydryl groups or of the hemochromogen absorption spectrum. In fact, all hemoglobin derivatives studied by Steinhardt revealed the same spectrum in water as in 4.4 M urea or 4.5 M formamide. The dissociated molecules retained their ability to combine reversibly with oxygen and carbon monoxide in the presence of the amides, though, as later work by Taylor and Hastings (383) showed, to a slightly diminished extent.

That some sort of change has occurred upon dissociation is revealed by the observation that, upon removal of the dissociating agent, the greater part of the protein failed to go in solution, whereas the soluble "regenerated" fraction was considerably less homogeneous in the ultracentrifuge than the native hemoglobin

⁹ 62,300 in 65 per cent glycerol (415).

(374). Also, urea-denatured hemoglobin is digested by papain at a rate forty times as great as the native protein (234).

The action of concentrated solutions of these amides on hemoglobin is in some respects milder than that of hydrochloric acid; or, as Steinhardt points out, under the influence of the amides "hemoglobin has gone one step toward denaturation".¹⁰ This difference in behavior may be explained by assuming that the dissociated globin undergoes further denaturation when the prosthetic group is split off, as it is by hydrochloric acid but not by urea. This may be quite similar to the difference in behavior of *Limulus* hemocyanin and *Cancer* hemocyanin toward the action of urea, already referred to in this section. These two types of dissociation of hemoglobin may be represented schematically by the following equations:

Acid dissociation:



and



where H_4 represents the heme moiety, Gl native globin, and Gl' denatured globin.

Urea dissociation:



It appears that the bond between heme and globin cannot be broken (by acids) without denaturation of the latter, and that this bond inhibits the denaturation of globin by urea and other amides.

(j) *Tobacco mosaic virus*: The literature on this subject has been excellently reviewed by Bawden and Pirie (52). Tobacco mosaic virus, potato "X" virus, tomato bushy stunt virus, and tobacco necrosis virus are irreversibly denatured by urea, with a concomitant loss of infectivity and serological activity. The kinetics of the urea denaturation of tobacco mosaic virus has been studied extensively by Stanley and Lauffer (224, 371). The streaming double refraction and the turbidity of the virus solution decreased progressively, in like manner, with time when the virus was treated with 6 *M* urea at pH 7. This was accompanied by a proportionate decrease in the amount of protein soluble after removal of urea by dialysis. Osmotic-pressure measurements indicated that the molecular weight of the protein was between 40,000 and 100,000, as compared to 40×10^6 for the native virus. This degradation of the protein upon urea denaturation was accompanied by the splitting off of nucleic acid.

In a recent investigation it was shown (354a) that 72 hr. exposure of tobacco mosaic virus to *M*/100 glycine buffer, pH 9, at 0°C., yields partial dissociation of the virus into two components, one of which is free of nucleic acid. Both

¹⁰ These considerations apply to the specific conditions of Steinhardt's experiments where the protein was exposed to up to 4.4 *M* urea solutions for periods of 1 to 4 hr. Drabkin (128) reports that upon prolonged contact 6 *M* urea may act on hemoglobin much in the same manner as does 0.008 *M* sodium hydroxide, 13 hr. being required for one-half completion of the conversion of horse oxyhemoglobin to hemochromogen.

components are homogeneous in sedimentation and electrophoresis, having a molecular weight of about 360,000. The nucleic acid-free fraction migrates at pH 9 with an electrophoretic mobility about 60 per cent lower than that of the unchanged virus, whereas the nucleic acid-containing component has the same mobility as the virus. Readjustment of the solutions containing either one of the biologically inactive dissociation products to pH 5 yields a material which resembles the native tobacco mosaic virus in sedimentation constant, crystal form, and electron-microscopic appearance. Also, solutions of these reformed virus proteins exhibit double refraction of flow, though the material is biologically inactive. It appears, therefore, that reassociation occurred by means of an end-to-end aggregation of about one hundred of the moderately asymmetric dissociation products, yielding a material which resembles the native protein in gross molecular properties but which is devoid of the details of internal configuration of the latter, as revealed by the lack of biological activity.

Denaturation by high pressures (222) leads at first to a disintegration into components of sedimentation constant $S = 35$, which are somewhat anisometric and do not seem to have lost an appreciable amount of nucleic acid. This inactive material is then further decomposed into insoluble protein, devoid of nucleic acid.

(k) *Myosin*: Weber's osmotic-pressure measurements indicate a reduction in molecular weight from about 1,000,000 to about 100,000 (400) in 6.66 *M* urea solution.

It has been concluded that the loss of double refraction of flow, produced also by guanidine and methylguanidine salts, arginine monohydrochloride, calcium and magnesium chlorides, potassium iodide, sodium thiocyanate, and other salts is likewise accompanied by splitting of the molecules into smaller fragments (133).

(l) *Pepsin*: When solutions of crystalline pepsin are brought to pH 7 or higher, the sedimentation constant decreases although the protein remains homogeneous. It is not certain whether this change is due to molecular dissociation or to a change in the degree of asymmetry or hydration. Readjustment of the pH of solutions of alkali-inactivated pepsin to a pH of 4.8 or less results in a rise in the sedimentation constant and in a decrease in the degree of homogeneity (323).

(m) *Diphtheria antitoxin*: Treatment of antitoxic pseudoglobulin with pepsin at pH 4.2 results in molecular dissociation, with the formation of half-molecules. Diffusion and sedimentation measurements indicate (322, 391) that cleavage occurred in a plane normal to the major axis of the molecule. The inactive pseudoglobulin contained in antitoxin preparations, purified by salting out and dialysis, is attacked by the enzyme in the same manner as the immunologically active component. However, the remaining components of antitoxic sera are digested into smaller fragments.

(n) *Insulin*: In the presence of 1 per cent Duponol, both native and reduced insulin have a lower molecular weight than in the absence of the detergent, a result suggestive of molecular dissociation (262). However, as pointed out by the authors, the possibility of a retardation of the sedimentation rate because of electrostatic interaction with the charged detergent micelles cannot be dis-

(o) *Helix pomatia hemocyanin*: The work of Brohult (72) reveals that *Helix pomatia* hemocyanin having a molecular weight of 8.91×10^6 may be split reversibly into components of about one-half and one-eighth of the native molecule. Calculations of molecular shape suggest that in both instances splitting occurs in a plane parallel to the major axis of the molecules (289). The degree of dissociation depends on the pH of the solution, half-molecules appearing in *acidic* pH ranges and both half- and eighth-molecules in *alkaline* regions. Recombination results in the restoration of the original molecular-kinetic properties; however, the recombined molecules differ from the native in electrophoretic mobility (393). The dissociation by ultrasonic waves, ultraviolet light, and α -rays has already been referred to and contrasted to the dissociation by chemical agents. Dissociation by electrolytes depends on the valence of the component anion and cation and on the ionic strength. Non-electrolytes, such as glucose and fructose, glycerol, and urea, are also capable of causing dissociation.

3. Denaturation causing aggregation of protein molecules

It is a matter of experience that denatured proteins are considerably more susceptible to aggregation than native proteins (73). The degree of aggregation is dependent on external conditions, such as pH, temperature, and ionic strength of the medium, and is governed by the electrokinetic potential on the protein particles. Although more observations have been reported on the aggregation than on many other properties of denatured proteins, little is known about the actual state of dispersion of the aggregates. Since aggregation may occur under conditions for which there is no evidence that they cause denaturation,—for instance, preparatory to precipitation of native proteins by salts,—it is not a phenomenon specific for denaturation. Thus, horse serum globulin has a particle weight of about twice that of the native protein when dissolved in 1 *M* ammonium sulfate at pH 4.3 (82), or in 2 *M* guanidine hydrochloride (296), without showing any evidence of being denatured. Myogen becomes aggregated in pairs when dissolved in 1.4 *M* ammonium thiocyanate, also without the appearance of any denaturing effects (401). Examples of aggregation accompanied by denaturation are the irradiation of serum proteins by ultraviolet light, whereby the viscosity is increased and the colloid osmotic pressure is decreased (123).

Ultracentrifugal measurements have been reported for the denaturation of human serum albumin by soft x-rays. The sedimentation constant of the undenatured fraction remains constant; however, as denaturation proceeds, an increasing proportion of the protein is converted into aggregates as well as into low-molecular-weight fragments (352).

When salt-free solutions of crystalline horse serum albumin are heated to 70°C., aggregation occurs (318), varying in degree with the pH of the solution (118). The mean molecular weight of the somewhat polydisperse protein was about four times that of the native when heat-treated at pH 7.6, about nine times at pH 4.3, and about twice when heat-treated at pH 3.6. These changes are

largely irreversible; in fact, further aggregation occurs when the solutions heat-treated at pH 7.6 and 4.2 are readjusted to the isoelectric point. A preliminary announcement by Rothen indicates somewhat similar reactions of heat-denatured egg albumin (347; see also 225).

The problem of the aggregation of denatured proteins does not invite detailed consideration as long as more quantitative data on the state of dispersion, particularly in relation to other changes in properties, are lacking.

B. MOLECULAR SHAPE AND THE QUESTION OF HYDRATION

It is beyond the scope of the present review to discuss the problem of the shape of native protein molecules. Recognition of differences in shape characteristics has led to differentiation between corpuscular and fibrous proteins, the former group comprising spherical and moderately anisometric molecules, the latter, molecules of rod-shaped and fibrous configuration (29). Under the influence of certain kinds of denaturing agents, globular proteins may be converted into the fibrous state, while fibrous proteins, such as myosin and tobacco mosaic virus, assume a more nearly spherical configuration.

The methods for determining the shape of protein molecules have been reviewed elsewhere (286, 289, 309) and will be discussed here only as they have been applied to the investigation of the denaturation process. Theoretically least ambiguous are measurements of the birefringence produced by the orientation of optically isotropic, anisometric particles by hydrodynamic forces (double refraction of flow). The ratio of the major to minor axis of prolate or oblate ellipsoids may be calculated from the rotatory diffusion constant, the latter being determined by measurements of the amount of double refraction and of the angle of isocline (*cf.* 113, p. 527 ff.). For very anisometric particles, the length of the major axis may also be estimated. Fairly accurate data are available for myosin, tobacco mosaic virus, *Helix pomatia* hemocyanin, and others (*cf.* 113, p. 527 ff.). Qualitative measurements of the changes in birefringence upon denaturation have been carried out on myosin (133, 282, 284, 400; also, *cf.* 113, p. 527 ff.) and tobacco mosaic virus (256, 371). Experimental difficulties of attaining a sufficiently high velocity gradient have impeded the general application of this method to less anisometric protein molecules.

By far the most common methods for comparing the shapes of native and denatured proteins are those based on the frictional ratio, f/f_0 , and viscosity measurements (286, 287, 289, 294, 309, 326). In the first of these, the molecular frictional coefficient effective in diffusion or sedimentation is compared to that expected for a spherical anhydrous molecule. The frictional ratio is a composite function of molecular asymmetry and hydration. Assuming a value for the latter, the former can be evaluated with the aid of the Perrin equation, and expressed in terms of axial ratios of prolate or oblate ellipsoids (294, 309). This method of estimation of molecular shape is attended with a certain degree of ambiguity, since (1) a model of ellipsoids of revolution may be an inadequate description of the shape properties of proteins, and (2) the degree of hydration cannot be evaluated by present-day methods.

The same limitations apply to viscosity methods where the limiting value of the intrinsic viscosity of the solutions $(\eta/\eta_0 - 1)/c$, measured under conditions which favor either complete orientation or random distribution of the solute molecules, is related to the ratio of molecular axes. Of the several equations that have been formulated, that of Simha (361) appears to be most satisfactory for proteins (294). With very anisometric molecules, the apparent viscosity varies with the rate of shear. At low rates it is appreciably higher than at high rates, since under the latter conditions the molecules become oriented, thereby requiring less energy for viscous flow. With spherical molecules, the viscosity is independent of the rate of shear (252). It has also been noted that in general with spherical molecules the relative viscosity increases linearly with solute concentration up to higher concentration ranges than it does with asymmetric molecules (262).¹¹

In the past, before the shape properties of proteins had received proper consideration, there was a tendency to interpret the observed increase in viscosity of proteins upon denaturation in terms of increased hydration and aggregation (15, 173, 180, 212). As will be shown presently (page 226), this viewpoint is no longer tenable, particularly in those cases in which there is no evidence that denaturation causes aggregation of molecules. Evidence from various sources suggests that the *changes* in intrinsic viscosity or in frictional ratio (dissymmetry constant) are results of changes in molecular shape.

1. Experimental results

The following proteins have been found to assume a more asymmetric shape upon denaturation by various means:

(a) *Serum albumin*: The specific viscosity of solutions of the crystalline protein increases with increasing urea concentration, while the diffusion constant decreases in proportion. A limiting value is approached in about 6 *M* urea solutions (300). Since the molecular weight of the protein remains unchanged (page 212), the changes in diffusion and viscosity properties have been taken as indications of alterations in shape. It is of interest to note that diffusion data reveal the solutions to be monodisperse when the urea concentration is higher than 0.5 *M*, whereas they should have been heterodisperse had the solutions contained mixtures of completely denatured and native protein molecules (270).

The increase in the degree of denaturation with increasing concentrations of the denaturing agents is paralleled by an increase in the fraction of insoluble, irreversibly denatured protein, obtained after removal of urea by dialysis under specified conditions (295). This suggests a physical correlation between the extent of distortion of intrinsic configuration of the molecules and the susceptibility of the denatured protein to irreversible changes. The fact that the water-soluble fraction (obtained after removal of urea) resembles the native protein in molecular shape (and size) shows that these globular proteins are endowed with a latent long-range elasticity, comparable in magnitude to that characteristic for certain fibrous proteins, i.e., keratin and myosin (29).

¹¹ Comparison of the viscosities of denatured and native proteins supports the validity of this empirical relation (118, 295, 296, 300).

In analogy with measurements of the chemical reactivity (Part II A), diffusion and sedimentation measurements have revealed a greater denaturing power of guanidine hydrochloride as compared with that of equimolar concentrations of urea. Thus, the axial ratio, calculated for a prolate ellipsoid of revolution and assuming 33 per cent hydration, was 3.3 for the native protein and 13.3 and 16.7 for serum albumin denatured by urea and by guanidine hydrochloride, respectively. Differences in the degree of denaturation were found between horse and beef serum albumin (331), and even between preparations of the latter when carried to different stages in the purification process. The effects of heat-treatment on the molecular shape of serum albumin are small as compared with those produced by urea, and conceivably may be accounted for by an end-to-end aggregation of molecules of unchanged shape (118). However, the conditions of these two sets of experiments are not strictly comparable, since in the latter instance measurements were performed in the presence of the denaturing agents, whereas in the former, they were carried out after protein solutions had been heated (at 70°C.) and cooled. No information is available on the shape changes occurring at the temperature of heating.

Ultracentrifugal experiments have furnished evidence that with one protein, i.e., thyroglobulin (245, 249), heat-treatment may produce shape changes of the type described for the urea denaturation of serum albumin.

(b) *Serum globulin*: The action of concentrated solutions of urea on this protein is similar to that on serum albumin (296). Denaturation by guanidine hydrochloride is a more complex process: in 2 *M* solutions the molecules become aggregated in pairs. In 3 *M* solutions of guanidine hydrochloride, the molecular assymetry is increased to the same extent as in 8 *M* urea solutions; however, the mean molecular weight is about one-half that of the native protein. Further increase of the concentration of guanidine hydrochloride, to 5.6 *M*, produces no further change in molecular shape or weight.

(c) *Myogen*: Sedimentation and diffusion data yield a dissymmetry constant of 1.26 for the native protein, and of 3.2 for the urea-denatured half-molecules (154). This corresponds to a change in axial ratio from 3.0 to 49.0. The action of urea on this protein is similar in nature to that of guanidine hydrochloride on serum globulin, though of considerably greater magnitude.

(d) *Hemoglobin*: Native hemoglobin appears to approximate in shape an oblate rather than a prolate ellipsoid of revolution (70). The dissociation products obtained by denaturation by acids have about the shape properties that would be expected to result from mere cleavage along the major molecular axis.

(e) *Egg albumin*: While several investigations have been recorded on the effects of denaturation on the viscosity of egg albumin solutions (15, 236, 239), most of these were performed in a limited but high concentration range in which the relative viscosity is no longer proportional to protein concentration. For this reason, and because no adequate precautions were taken to repress electroviscous effects, the data do not lend themselves to quantitative interpretation and will not be considered here.

Quantitative viscosity measurements have been carried out by Bull (75) on solutions of crystalline egg albumin denatured (1) by heating at pH 8.0 for 7 min. at 100°C., followed by dilution with phosphate buffer (pH 8.0), and (2) by adding 1 g. of urea per cubic centimeter of protein solution and, after allowing the material to stand for 1 hr. at room temperature, diluting with phosphate buffer. The viscosity increase produced by heat denaturation was smaller than that observed after denaturation by urea. This result is in keeping with the comparative effects of these two types of denaturation on the sulphydryl groups of egg albumin.

(f) *Helix pomatia hemocyanin*: The shape changes accompanying dissociation and denaturation (72) have been referred to (Part II A). Calculations of dissymmetry constants and axial ratios suggest that cleavage occurs along the major axis without any further elongation of the dissociation products (289).

Myosin and tobacco mosaic virus exemplify conditions where denaturation causes a change in the shape of anisometric molecules toward a more globular configuration. It is well to stress, however, that these changes may be the result of the dissociation of these protein molecules, occurring with both proteins in the presence of concentrated solutions of urea (pages 218, 219).

(g) *Myosin*: Von Muralt and Edsall (282) found that urea in concentrated solutions, and iodides and thiocyanates in dilute solutions, abolished the double refraction of flow. A variety of other agents was found by Edsall and Mehl (133) to produce similar effects, varying in extent with the nature and concentration of the substances they have studied. These include chlorides of monovalent and divalent cations, ammonium, methylammonium, and lithium salts, guanidine and methylguanidine salts, urea, and arginine hydrochloride. The double refraction of flow was also irreversibly lost upon acidification, contrary to the earlier results of Bate-Smith (44), as well as by heating. The decrease in double refraction was paralleled by a decrease in relative viscosity, indicating that the protein molecules had assumed a less asymmetric configuration.

(h) *Tobacco mosaic virus*: Denaturation of this virus protein by urea follows closely the pattern already described for myosin. The double refraction of flow decreases as denaturation progresses (256, 371). The relative viscosity of virus protein solutions falls off sharply, and viscosity anomalies are greatly reduced (145, 371). These findings, together with the observations of a decrease in osmotic pressure and sedimentation constant (224, 371), are indicative of changes in the direction of less anisometric shape, and disaggregation. Urea denaturation causes inactivation of the virus and an alteration of serological specificity (52). Old virus preparations, isolated by salt fractionation, reveal a tendency to aggregate to products which are more anisometric than the untreated protein. Such changes are elicited by variations of pH from pH 6.8 to pH 4.5 (256).

The effect of native and of denatured proteins on the physical properties of sodium thymus nucleate

Aqueous solutions (0.1–0.5 per cent) of sodium thymus nucleate are highly viscous and possess intense double refraction of flow (170). These properties are related to the highly asymmetric shape of the nucleate particle. The latter has been estimated to possess an axial ratio of 300 (360) and a length of 450 m μ (385). When solutions of the nucleate are treated with salts, amino acids, or proteins, the birefringence and viscosity of the nucleate diminish, and the extent of the diminution in these properties is a function of both the nature and the concentration of the added substance (170). Guanidine salts are highly effective in this respect, and the similarity of their effect upon the nucleate and upon the protein myosin (133) is indeed noteworthy. On a molar basis, the most powerful agents in reducing the viscosity and intensity of streaming birefringence of the nucleate are the *native* proteins (170). When the proteins are *denatured* prior to adding them to the nucleate, the former possess relatively little effect upon the physical properties of the nucleate. This is indeed striking, for in this phenomenon it is the native protein which is reactive and the denatured protein which is relatively inert. The effect of the protein on the properties of the nucleate through the free groups of the protein as such must be of a secondary nature; the specific configuration of the *native* protein is probably of primary importance in the effect on the nucleate. This is not wholly unexpected when it is recalled that a common method of separating nucleic acid from protein in the native nucleoprotein molecule involves the denaturation of the latter (166). There is little knowledge available concerning the nature of the interaction of native protein and nucleate. The latter in such mixtures loses its osmotic effectiveness and probably aggregates into somewhat symmetrical, large bundles (165).

It has been noted that the sedimentation constant of several native globular proteins is decreased in the presence of other native proteins (381). Since this may have been caused by changes in molecular shape rather than in molecular weight, it appears that these effects are not confined to sodium thymonucleate but are manifestations of a general denaturing action of native proteins.

2. General considerations

Values of the frictional ratio, f/f_0 , greater than unity may be due to the combined effects of hydration and asymmetry. This may be expressed formally by the relation (309):

$$f/f_0 = (f/f_e) (f_e/f_0)$$

where the first term denotes the hydration factor and the second term the asymmetry factor, the latter being related to the ratio of molecular axes by the Perrin equation (286).

Similarly, the intrinsic viscosity, $(\eta/\eta_0 - 1)/c$, is a composite function of molecular volume and asymmetry, according to the relation:

$$\frac{\eta/\eta_0 - 1}{cV_h} = \varphi(b/a)$$

where c is the protein concentration in weight per cent, V_h the partial specific

volume of the hydrated protein, and ϕ a function of the axial ratio. V_h is related to the degree of hydration according to:

$$V_h = (rV_1 + V_0)$$

where V_0 is the partial specific volume of the anhydrous protein, V_1 that of the solvent, and r the number of grams of water combined with 1 g. of anhydrous protein. An increase in specific viscosity may be due to changes in r , in ϕ , or in both.

It is generally agreed that protein molecules are hydrated in solution as well as in the solid state (157) and that the degree of hydration may vary within certain limits with changes in environmental conditions. While there is no unambiguous method for determining the state of hydration, various lines of evidence suggest a value of the order of 30 per cent (73, 157, 292, 309). The present problem is not concerned with the actual value but with the *changes* in hydration upon denaturation.

Correlation of the following experimental facts leads to the conclusion that these changes are negligible in comparison to the changes in molecular asymmetry, i.e., *the increase in viscosity and frictional ratio is largely, if not wholly, due to changes in molecular shape*:

(1) If the decrease in diffusion constant and the increase in intrinsic viscosity of serum albumin upon denaturation by 8 *M* urea were to be interpreted solely by a combination between protein and urea, solvation to the extent of about 2.7 g. of urea per gram of protein would have to be assumed, equivalent to about 3200 urea molecules per protein molecule (295). This would lead to the rather unreal result that in a 1 per cent protein solution one-third of the urea molecules are bound by the protein, and in a 3 per cent solution, all of them. If solvation is assumed to remain constant, a change in axial ratio from 3.3 to 13.3 is calculated (table 10). That the latter interpretation is more nearly correct is evidenced by a comparison of the frictional ratio of egg albumin in the presence of urea (406) with the specific viscosity of urea-denatured egg albumin in aqueous buffer solutions (75). The agreement is very satisfactory, indicating that the changes in properties were not prompted by the adsorption of urea molecules but rather by the effects of the latter on the molecular shape of the protein.

(2) The intrinsic viscosity of serum albumin is increased by synthetic detergents (Duponol, sodium dodecylsulfate, etc. (247, 332)). A 0.035 *M* solution of sodium dodecylsulfate is about as effective as an 8 *M* urea solution. Higher concentrations of detergents are even more effective on both the acid and the alkaline side of the isoelectric point of the protein (332). Since under the conditions, not sufficient detergent molecules are present to account for the increase in intrinsic viscosity in terms of adsorption of detergent by protein, one has to assume either that water molecules are adsorbed or else that the effects are due to changes in molecular asymmetry. The initial unequal distribution of urea between the interior of the protein molecules and the surrounding medium conceivably might cause an osmotic swelling, resulting in the penetration of the protein by urea and water molecules. However, such a mechanism is unlikely.

to underlie the interaction between detergent and protein, since (1) the detergent molecules would be too large to penetrate the protein, and (2) detergent is present in too low concentrations to exert appreciable osmotic effects.

(3) When proteins are denatured by urea or by detergents, they may be spun into fibres by forcing the solutions through narrow orifices into suitable media (35, 247, 404).

The assumption that visible fibre formation is only a result of changes in the solubility of the fibrous denatured proteins is certainly more realistic than any hypothesis which would have to postulate that hydrated spherical molecules unfold into fibrous structures upon changing the solvent composition. Moreover, there is one kind of denaturation, i.e., that by surface forces, where the conversion of native globular proteins to the fibrous denatured state has been definitely established (Part III D).

(4) Comparison of the x-ray diffraction patterns of native and denatured proteins, to be discussed in greater detail in Part III D, indicates the latter to yield a diffraction pattern resembling that observed with several fibrous proteins. Again, this would be unlikely to be the case if denaturation were to result merely in an increased hydration of the molecules.

(5) Heat-coagulated and surface-coagulated egg albumin have higher densities than the native protein when xylene is used as displacing agent (292), whereas the densities of these three forms are about the same when hydrogen is used as displacing gas (73). Although the degree of hydration is undoubtedly different in the coagulated state from what it is when the proteins are dissolved, the lower densities observed for the denatured proteins in the presence of toluene indicate that they have a denser and therefore less penetrable structure than the native protein (73).¹²

(6) The solubility of proteins is lower in the denatured than in the native state. This would hardly be expected if denaturation were to increase the hydration of essentially spherical molecules, whereas it can be accounted for by an increase in asymmetry accompanied by a redistribution of polar and non-polar groups.

In the light of these considerations one is led to the conclusion that, in all cases considered, the changes in viscosity and frictional coefficient are brought about by changes in molecular asymmetry. Indeed, they constitute a significant criterion for protein denaturation. In table 11 data are given for the shape changes of representative proteins, calculated with the assumption of about 33 per cent hydration, and expressed in terms of a ratio of the major to minor axis, b/a . With the exception of hemoglobin, the molecules have been assumed to approximate the shape of prolate ellipsoids.¹³ The degree of change in shape

¹² Dilatometric investigations also indicate denatured proteins to be less hydrated (186).

¹³ General interpretation of the shape changes in terms of flat ellipsoids has not been attempted, nor does it seem warranted at the present time. Although it has been suggested (27) that certain native proteins, such as egg albumin, consist of superposed laminae, which are separated by side chains and are liberated upon denaturation (30), there is no unequivocal evidence to sustain this hypothesis.

is seen to depend on the nature of the denaturing agent and on the susceptibility of the protein to its action.

TABLE 11
Shape changes accompanying protein denaturation

PROTEIN	DENATURING AGENT	LONG AXIS ^(a) SHORT AXIS		CALCULATED FROM ^(c)	REFERENCE
		Native	Denatured		
Serum albumin (horse).....	1.5 <i>M</i> urea	3.3	4.1	f/f_0	(300)
	3 <i>M</i> urea		5.3	f/f_0	(300)
	6 <i>M</i> urea		13.3	f/f_0	(300)
	8 <i>M</i> urea		13.3	η	(295)
	8 <i>M</i> guanidine hydrochloride		16.7	η	(295)
Serum globulin (horse).....	5 <i>M</i> urea	5.2	9.3	η	(296)
	8 <i>M</i> urea		15.4	η	(296)
	5.6 <i>M</i> guanidine hydrochloride		15.0	η	(296)
Myogen.....	Urea	3.0	49	f/f_0	(154)
Egg albumin ...	Heat	3.1	5.5	η	(75)
	Urea		7.1	η	(75)
Hemoglobin.....	Acids	2.7 ^(b)	6.4 ^(b)	f/f_0	(155, 289)
<i>Helix pomatia</i> hemocyanin ^(d) ..	pH change	2.7 ^(e)	8.3 ^(f) 11.0 ^(g)	f/f_0 f/f_0	(72)

^(a) Unless otherwise stated, values have been calculated for prolate ellipsoids of revolution, assuming 33 per cent hydration.

^(b) Oblate ellipsoid of revolution.

^(c) f/f_0 = calculation from the dissymmetry constant with the Perrin equation (286).

η = calculation from viscosity data with the Simha equation (361).

^(d) Asymmetries calculated from f/f_0 and double refraction of flow (72).

^(e) $M = 8.9 \times 10^6$.

^(f) $M = 4.31 \times 10^6$.

^(g) $M = 1.03 \times 10^6$.

C. ELECTROCHEMICAL PROPERTIES

1. Titration curves

Comparative measurements of titration curves have been used to determine the influence of denaturation on the amphoteric properties of native proteins. Although the results obtained are of some interest, the following considerations render their interpretation difficult: (1) The insolubility of denatured proteins near the isoelectric point frequently necessitates recourse to heterogeneous titrations, the latter being of doubtful significance. (2) While maximum acid and base combination takes place only in regions of extreme acidity (pH 1-2)

or alkalinity (above pH 12-13) (*cf.* 113, p. 444 ff.), many proteins are already denatured in these regions. Hence, comparison of these quantities with "native" proteins is impossible; moreover, in these extreme regions, titrations are least dependable. Methods for the detection of such irreversible changes during titration have been reviewed by Cannan (87).

Difficulties attending comparison of the complete titration curves of native and denatured proteins are best illustrated by hemoglobin. Titration with acids below pH 4 results in rapid denaturation, even at room temperature (67, 121), as witnessed by the fact that below this pH the titration curve cannot be reproduced by back titration with alkali (*cf.* 113, p. 444 ff.). Therefore, it is not surprising that early workers (229) reported the titration curves of allegedly native and denatured hemoglobin with acids to be identical in this region. Though other proteins may react reversibly with acid and base over a wider range (pH 1.5 to pH 11-12) (87), they are readily denatured beyond these limits (120, 121).

There is much confusion in the literature regarding even that portion of the titration curves of native and denatured proteins which lies within the pH-stability range. While some workers variously claim that the titration curve of heat-denatured egg albumin differs from that of the native (101, 182, 411, 413), others assert their identity (67, 240, 330). In part, the contradictory claims of different workers may be reconciled by correcting for the alkaline pH shift attendant on denaturation, the titration curves of native and of heat-denatured egg albumin becoming identical if calculated from a common isoelectric point (pH 4.7-4.8) (101, 240). The invalidity of this procedure will be discussed shortly.

Not only do the titration curves given by various workers for denatured egg albumin differ from one another, but also the curves for the native protein are often inconsistent with those given in recent investigations (120; also *cf.* 113, p. 444 ff.). Accordingly, these results do not merit detailed analysis. Suffice it to say that any changes in amphoteric properties that may occur on denaturation are of a small order of magnitude.

Recently, the titration curve of untreated collagen has been compared with those of collagen denatured by salts (288) and by heat (387), with the unusual result that a shift in the isoionic point was observed in opposite directions for the two modes of denaturation, a phenomenon not amenable to ready explanation.

2. Isoelectric point and electric mobility

It has been shown that in solutions of the same ionic strength the electric mobility of a protein at different hydrogen-ion concentrations is directly proportional to the number of hydrogen or hydroxyl ions bound (1). Accordingly, the electric mobility curves are superimposable on the titration curves upon proper selection of the ordinates, thus providing another means for the comparison of the amphoteric properties of native and of denatured proteins, as well as for the determination of isoelectric points. The application of the electrophoretic technique to the study of denaturation has already been reviewed (1) and will be discussed here only in brief.

(a) *Egg albumin*: The data of Moyer (281) indicate that the isoelectric point of surface-denatured egg albumin is pH 5.02 under the same conditions for which the dissolved native protein is isoelectric at pH 4.55 (390). The occurrence of an alkaline shift at the isoelectric point of denatured proteins is substantiated by the pH increase observed in the surface (78, 412), heat (73), and urea denaturation (413) and the ultraviolet irradiation (62) of egg albumin. In the light of these findings, comparison of the titration curves of native and of heat-denatured egg albumin, by recalculation of the data to a common isoelectric point, does not appear valid.

The electrophoretic mobility curve of surface-denatured egg albumin is displaced 0.47 pH unit to the alkaline side relative to that of the native protein, but the curves remain parallel over the limited pH range investigated (1, 281).¹⁴ In keeping with these results, the mobility of completely heat-denatured or acid-denatured egg albumin on the alkaline side of the isoelectric point likewise is slightly less than that of the native form (237).

(b) *Serum albumin*: Heat denaturation of horse serum albumin brings about an alkaline shift in the electric mobility curve quite analogous to that observed in the surface denaturation of egg albumin (318). However, regeneration of urea- or detergent-denatured horse serum albumin displaces the mobility curve to the acid side above pH 5. Thus, while urea regeneration results in an increase in electric mobility above pH 5 (358), as does the regeneration of detergent-treated protein at pH 7.6 (332), heat denaturation brings about a decrease in mobility in the same pH region (318). Yet, under certain conditions, two components may be distinguished in heat-denatured horse serum albumin (118), one of lower mobility than the native (perhaps corresponding to irreversibly denatured protein) and one of greater mobility (perhaps identical with regenerated albumin). However, it is remarkable that the ratio of components may be greatly altered merely by changing the ionic strength.

On the acid side of the isoelectric point, heat denaturation of horse serum albumin results in the formation of but a single component, this migrating more rapidly than the native albumin (118, 318). At pH 3.6 urea-regenerated albumin likewise migrates somewhat faster than the native protein (358).

The results for urea regeneration of beef serum albumin are in accord with those for horse serum albumin, a small increase in mobility being experienced for the regenerated protein at pH 7.6 (331). On the other hand, at the same pH the mobility of bovine albumin regenerated by guanidine hydrochloride (299, 331) is identical with that of the native protein. However, the mobility of albumin irreversibly denatured by guanidine hydrochloride is less than that of the native or regenerated protein (299), in agreement with the results already quoted for heat-denatured horse serum albumin.

(c) *Other proteins*: Acid-denatured hog thyroglobulin behaves similarly to

¹⁴ In contrast to the identical behavior usually observed for the dissolved and adsorbed proteins, the mobility curve of adsorbed egg albumin likewise undergoes a small shift to the alkaline side, the isoelectric point changing to pH 4.82. It is suggested that egg albumin may become "partially denatured" during the process of adsorption (1).

surface-denatured egg albumin and heat-denatured horse serum albumin in that the electric mobility curve remains parallel to that of the native protein but is transposed about 0.4 pH unit toward the alkaline side at the isoelectric point (179). Likewise, the electric mobility of urea-regenerated horse serum pseudoglobulin is higher on the acid side and lower on the alkaline side of the isoelectric point (359).

The electrophoretic properties of denatured proteins are thus consistent with their titration behavior in that they indicate that only small changes in amphoteric properties occur as a result of denaturation. However, while the titration data are ambiguous, the electrophoretic data are unequivocal in indicating an alkaline shift in the acid- and base-binding properties of the denatured protein.¹⁵

One explanation for the shift in the isoelectric point upon denaturation may be found in the theory of Mirsky and Pauling (278), according to which denaturation results from the rupture of hydrogen bonds, the latter serving to maintain the intact native structure. Since the acid-base properties of a protein are determined primarily by the groups which are free, the liberation of paired amino and carboxyl groups in equal numbers should shift the isoelectric point towards neutrality.

Alternatively, since the strength of the acidic and basic groups in a protein molecule must depend somewhat upon the arrangement and proximity of neighboring groups, a profound alteration in molecular structure as a result of denaturation could suffice to explain a change in amphoteric properties even in the absence of the liberation of new titratable groups.

Bull (73) has discussed the small increase in acid-binding capacity observed upon denaturation in terms of both explanations: namely, (a) an actual increase in the number of proton-accepting groups, and (b) an increase in the binding strength of such groups.

3. *Electrophoretic homogeneity*

Although denaturation leads to an increase in molecular-kinetic heterogeneity as a result of dissociation and aggregation, the denatured proteins may yet retain the electrochemical homogeneity of the native protein. Indeed, protein mixtures may undergo an increase in electrical homogeneity upon denaturation. Egg albumin, whether denatured by heat or by acid, is electrically homogeneous at pH 6.8 and pH 10.28, but two components may be observed after prolonged dialysis at pH 12.81, the slower component apparently representing the denatured form (237). Heat-denatured serum albumin is electrophoretically homogeneous on the acid side of the isoelectric point, two components arising on the alkaline side (118).

In contrast to the splitting and aggregation often occurring upon the denaturation of proteins, it is surprising to observe that the denaturation of horse

¹⁵ It is interesting to note that hemocyanins, dissociated into eighth-molecules outside the pH-stability range, form reassociation products at neutral pH, similar to the original molecules in molecular weight but distinguished by a 5 to 10 per cent decrease in electrophoretic mobility (393).

serum leads to an increase in electrophoretic homogeneity. Van der Scheer, Wyckoff, and Clarke (397) have found that heating of serum at 65°C. for a short time results in the progressive formation of a single denatured electrochemical component, "C," at the expense of the albumin and the globulins. This finding has been confirmed by Davis, Hollaender, and Greenstein (123), who have also observed the same phenomenon for ultraviolet irradiation. The denatured component exhibited approximately the mean mobility of the native serum. A decrease in colloid osmotic pressure accompanies the increase in electrophoretic homogeneity observed upon ultraviolet irradiation. In explanation of this phenomenon it has been suggested that ultraviolet irradiation first involves an unfolding and then a splitting and aggregation, producing molecules of similar charge distribution but of widely varying particle size (123).¹⁶

A marked alteration in electrophoretic pattern also occurs upon the photo-oxidation of immune rabbit and horse serum (363). Whereas the heating of individual solutions of casein or of antibody globulin produces no appreciable change in electrophoretic homogeneity, heat treatment of mixtures leads to a change in electrophoretic pattern, possibly owing to protein-protein interaction (205).

Urea-regenerated (358) and detergent-regenerated *horse* serum albumin (332), and urea-regenerated and guanidine hydrochloride-regenerated (331) *beef* serum albumin all approach the native protein in electrophoretic homogeneity. The irreversibly denatured, as well as the regenerated, form of *beef* serum albumin may be obtained in an electrochemically homogeneous state (299). Normal *horse* pseudoglobulin also gives an electrophoretic pattern nearly identical with that of the urea-regenerated protein (359).

D. STRUCTURE OF DENATURED PROTEINS: EVIDENCE FROM X-RAY AND SURFACE-FILM STUDIES

1. X-ray structure

Throughout this discussion it has been inferred that the primary effect of denaturation is an intramolecular rearrangement of the proteins. In this section we shall examine the evidence which x-ray diffraction measurements have furnished for changes in molecular structure. The pioneer work of Astbury and his associates on the structure of fibrous proteins will serve as a starting point.

The simplest diffraction pattern is revealed by silk fibroin (24-26). It can be interpreted in terms of oriented, fully extended polypeptide chains, separated by a backbone spacing of 4.6 Å. and by a side-chain spacing of about 9.5 Å., and there is little doubt that this interpretation is correct (76, 119). A similar pattern, although somewhat more diffuse, is obtained from fibres of keratin (24, 29, 37) or myosin (28, 34) by stretching them along the fibre axis; in the natural state, or rather after preliminary orientation by slight extension, these fibres yield patterns characterized by a spacing of 5.1 Å. along the fibre axis, and by

¹⁶ Recently, it has been reported (174) that the effects of heat on the electrophoretic behavior of bovine serum may be repressed by previous saturation with glucose.

side-chain spacings of 9.8 and 27.Å., the back-bone spacing being absent altogether.

The transition of both myosin and keratin from the natural to the stretched state is, from the viewpoint of x-ray structure, a denaturation and, indeed, the least complex type of its kind. The stereochemical structure of the denatured protein is fairly well established (β configuration), while that of the native protein is open to more than one interpretation (α configuration). Astbury and Bell (32) and Huggins (194) have suggested structural schemes which satisfy bond-distance and valence-angle requirements (see also reference 55), but the final word has not yet been spoken (*cf.* 76, 291). This purely mechanical denaturation derives its apparently reversible mechanism from the strains that are imposed upon cross linkages between adjacent polypeptide chains, these cross linkages probably being established by disulfide groups of the cystine moiety (315, 365), and by ion and dipole attractions between side chains (76, 95).

A more profound type of denaturation occurs when keratin or myosin is exposed to higher temperatures or to alkali. With myosin, certain of the cross linkages are broken spontaneously in hot water or dilute alkali. In this state a diffraction pattern is observed which has been ascribed to disoriented fibres of the β configuration. With keratin, supercontraction occurs *after* the fibres are stretched, exposed to steam or alkali, and then allowed to contract. However, if the fibres are retained in the stretched state, new cross linkages¹⁷ are formed and the fibres become set permanently in a fully extended configuration, yielding again a diffraction pattern of the β type. The greater susceptibility of myosin to supercontraction has been ascribed to the presence of fewer and probably more reactive cross linkages.

Astbury (30) has tabulated the amino acid composition of keratin and myosin. Though the data are somewhat incomplete and therefore tentative, they serve to show that keratin has about eight times as many cystine residues as myosin, and three times as many serine residues, both groups being influential in the formation of cross linkages, the former by virtue of covalent bonds, the latter by means of hydrogen bonds. If this is compared to the composition of silk fibroin, containing mostly glycine, alanine, and serine (44, 25, and about 14 per cent, respectively), the profound differences in reactivity and latent elasticity to which these three fibrous proteins are subject can only be ascribed to the influence of side-chain interaction.

The changes which myosin undergoes upon *dehydration* are not detectable by x-ray measurements (34). They result in aggregation of the α -form by further interaction between side chains of adjacent polypeptide chains. No sulfhydryl groups become exposed under these conditions (265-267), in contrast to the *denaturation* by heat, where the α configuration breaks down to give way to chain bundles of incompletely oriented fibres of the β configuration.

This brief survey of the intramolecular rearrangement of keratin and myosin has been given to emphasize one basic fact, i.e., that *the fibrous proteins, like the*

¹⁷ The importance of covalent cross linkages in conferring elasticity on wool is clearly illustrated by the work of Harris and associates (147, 315).

globular proteins, are subject to denaturation by mechanical and chemical forces, and that the degree of resulting disorientation is related to the reactivity and mutual interaction of the side chains.

Side-chain interaction is more profound in the case of the *globular* proteins, if for no other reason, because the coiled configuration of the polypeptide chains affords a closer geometrical proximity of side chains. This upon denaturation conceivably may lead to either one of two opposing effects: (1) a rupture of cross linkages, leading to the liberation of extended, though disoriented, polypeptide chains, of the kind observed with supercontracted myosin or keratin; (2) the formation of new cross linkages, as in the case of the permanently "set" keratin fibres, with the net result that the molecule has passed from one rigid configuration to another, less specific one.

Remarkable progress has been made in recent years in the investigation and interpretation of x-ray diagrams of crystalline globular proteins (*cf.* reference 142). A great number of very definite reflections have been observed for hemoglobin, lactoglobulin, insulin, and other proteins when in the wet state, while upon drying many of the smaller spacings disappear.

Investigation of the denatured proteins has been less satisfactory, in regard to both the conditions of denaturation and the resolution of the diffraction patterns. Following the work of Astbury and Lomax (36), Astbury, Dickinson, and Bailey (35) investigated the denaturation of edestin, excelsin, and egg albumin by urea and heat. In a typical experiment, edestin was denatured by dissolving it in aqueous urea, and the urea solution was poured evenly over a glass slide which was then inserted in water until the protein was deposited as a thin layer. After the protein had dried, the process was repeated several times and the accumulated layers of denatured protein were peeled off, compressed between glass plates, and cut into strips. These films were extensible up to 200–250 per cent of their original length, and when released contracted to about 100 per cent of their initial length. In the unstretched state, urea-denatured edestin gave a pattern similar to that of disoriented β -keratin fibres (supercontracted) while upon stretching, orientation became more complete, the resulting diffraction diagram resembling closely that of fully extended β -keratin. It is of interest to note that in this, as well as in all other experiments that have been recorded, denaturation of these globular proteins gave rise to a sharpening of the backbone spacing of about 4.6 Å. and to the appearance of at least one outer ring corresponding to a spacing of about 3.7 Å. (31), the significance of which is not quite clear (table 12).

While the aforementioned experiments were carried out on mechanically oriented *layers* of denatured proteins, more recent work on egg albumin and other proteins, denatured by anionic detergents (314a) or heat (356a), reveals the diagonal spacing of about 3.7 Å. to appear even prior to mechanical stretching of the bulky *precipitate* of denatured protein. Stretching serves mainly to improve the orientation of the polypeptide chains, yielding a pattern similar to that observed by Astbury for layers of denatured proteins and for β -keratin, alike. A summary of the most important diffraction spacings of denatured

globular proteins is given in table 12. The agreement between the data obtained by different workers may be seen to be extremely good.

In general, one may conclude that denaturation of globular proteins results in a structure similar to that revealed by keratin when it "supercontracts," i.e., a bundle of disoriented polypeptide chains. The interesting point, however, is that this configuration has been reached in these two instances from opposite ends, i.e., from a fibrous state with keratin (or myosin) and from a more condensed, globular state with edestin, egg albumin, serum albumin, etc. When the denatured proteins become oriented, as by stretching, a structural pattern, analogous to that of fully extended β -keratin fibres is obtained. This latter has been shown most clearly by investigations of the x-ray properties of built-up monolayers of egg albumin (53).

TABLE 12
X-ray diffraction spacings of some native and denatured proteins

PROTEIN	DENATURING AGENT	SIDE-CHAIN SPACING	BACKBONE SPACING	DIAGONAL SPACING	REFERENCE
		$\text{\AA}$.	$\text{\AA}$.	$\text{\AA}$.	
Egg albumin.....	Native	10.65	4.75		(31)
	Heat	10.2	4.75	3.67	(31)
	Heat	10.2	4.66	3.75	(314a)
	Heat	9.8	4.65	3.75	(356a)
	Alkyl benzenesulfonate	10.3	4.65	3.76	(314a)
Serum albumin.....	Native	9.7	4.6		(36)
	Heat	9.6	4.5	3.6	(36)
Edestin.....	Urea	10.0	4.5	3.7	(35)
β -Keratin.....		9.8	4.65	3.75	(36)

Lately, it has been reported that while fibrinogen can be made to change its pattern from one resembling α -keratin to that of the β configuration by a special method of lateral squeezing, no change in diffraction pattern occurs when fibrinogen is converted to fibrin (40). Thus, clotting does not appear to be an example of true denaturation.

Several qualitative studies on the changes in x-ray patterns of proteins upon denaturation have been reported (102, 368). However, these were not only done under ill-defined conditions of denaturation, but unfortunately on vacuum-dried preparations. Since the specific configuration of the globular proteins changes readily upon drying much in the same manner as it does upon denaturation (31), an evaluation of the meaning of these investigations is practically impossible. Spiegel-Adolf and Henny (368), in one of their investigations, claimed to have obtained evidence for the reversal of the heat coagulation of serum albumin. However, examination of the published transmission curves reveals a closer relation between native and coagulated protein, than between native and "reversibly" denatured protein.

The interpretation of x-ray data of denatured proteins in terms of a liberation of polypeptide chains receives strong support from measurements of the properties of protein monolayers. Since both types of measurements, though of quite different nature, lead to essentially the same conclusion, we shall, at this point, examine the evidence which measurements of protein films have provided:

2. Monolayers as prototypes of completely denatured proteins

A few years ago, Neurath and Bull (293) undertook a critical examination of the available data concerning the structure and properties of protein monolayers. It was concluded that under the influence of surface forces, protein molecules unfold when spread onto the surface of a water layer, or in an interface, and that the structure of these films corresponds closely to that predicted for an array of fully extended polypeptide chains of the β configuration (*cf.* 279). At low pressures, the chains were assumed to lie on their side, with the side chains parallel to the water surface, whereas upon lateral compression they were assumed to turn around until the side chains were nearly vertical. Evidence for this interpretation was derived from a comparison of mean surface area per residue and of film thickness, with the values calculated for fully extended polypeptide chains.

Further, it was concluded that in protein monolayers the polar side chains are oriented toward the aqueous phase, and the non-polar side chains toward the air phase. This, however, could only be so if along the *entire* polypeptide chains the side chains are alternately polar and non-polar, ($\dots$ PNPNPNPNP $\dots$), whereas a single deviation from this regular sequence, i.e., ($\dots$ PNPNP~~P~~PNP $\dots$), would result in one-half or more of the polar chains emerging from the upper surface of the film, and the corresponding fraction of the non-polar chains from the lower surface (74). There is good reason to believe that polypeptide chains will tend to assume a selective orientation in order to satisfy the respective affinities of polar and non-polar side chains for polar and non-polar media. Measurements of the adhesion tension of monolayers of egg albumin at various pressures (74) substantiate this view. Thus the films convert from a quite hydrophilic state at pressures below 0.5 dyne to a more hydrophobic state at pressures of about 1.5 dynes, owing to the forcing out of hydrophobic groups from the water surface. However, there is no need to conclude that this orientation is perfect; more often than not a hydrophobic group may remain in the water phase if the attainment of selective orientation should interfere with the steric arrangement of the chains.

This raises the question whether and to what extent a fully extended polypeptide chain is capable of internal rotation. Cockbain and Schulmann (109) suggested that separation of polar from non-polar side chains takes place by means of looping of the polypeptide chain, the mode of looping depending on the relative proportion and sequence of polar and non-polar groups. This part of their hypothesis is fully acceptable, provided that a proper rôle be accorded to the individual side chains as regards both mutual attraction (30, 76) and steric inhibition (288, 291). However, their conclusion that proteins cannot

consist of fully extended polypeptide chains of the β -keratin type, if the concept of kinking and looping is accepted, may be misleading, since by and large, and particularly from the x-ray viewpoint, it is more or less immaterial whether a long polypeptide chain is fully extended or whether it contains a few isolated folds. This is borne out by Astbury and Bell's (33) investigation of the x-ray structure and optical properties of built-up monolayers of egg albumin. To quote these authors: "The simplest description of the x-ray photographs so far obtained is that they are analogous to those of keratin that has been squeezed laterally in steam; this treatment not only brings about the α - β transformation, but also tends to orient the side chains perpendicular to the plane of flattening." By a simple mechanical device, Astbury and Bell were able to measure directly the thickness of films composed of up to 1764 monolayers, yielding a mean thickness per monolayer of 9.5 Å., in excellent agreement with surface-film measurements and with the value of the side-chain spacing of β -keratin. As is to be expected, unfolding of the protein molecule incidental to surface denaturation results in the liberation of the total complement of sulfhydryl groups of egg albumin (271).

The influence of denaturation on the spreading properties of proteins has been the subject of several investigations. The original observation that heat denaturation of serum albumin inhibited the formation of surface films at pH 4.4 (285) has been confirmed (74). The apparent lack of spreading power appears to be due to the aggregation of the heat-treated protein at this particular pH (118), since in lower pH regions spreading is not inhibited. This is true for both heat- and urea-denatured egg albumin which, at pH 2.2, exhibit the same film areas and compressibilities as the native protein (74). The two denatured proteins differed from each other and from the native material in their pH-film area relation. Stållberg (369) found also native and heat-denatured serum albumin to spread to about the same extent on a $M/150$ phosphate buffer, pH 7.0, when dispersed in an aqueous solution of 60 per cent propyl alcohol containing also 0.5 M sodium acetate. It appears, therefore, that a denatured protein unfolds under the influence of surface forces as readily as the native protein, if not more so, provided aggregation is avoided.

Langmuir (219) and Schaefer (353) reported differences between native and denatured proteins with respect to the "expansion patterns" they yield upon applying indicator oil on the protein monolayers. The meaning of these purely qualitative observations is obscure.

3. *Biological activity of protein monolayers*

Since surface denaturation of globular proteins results in a more or less complete liberation of fully extended polypeptide chains, it affords excellent conditions for determining the contribution of the intrinsic structure of the intact molecule to the biological activity of the protein. Unfortunately, in earlier investigations the precision requisite for obtaining truly monomolecular films has often fallen victim to the enthusiasm with which these experiments were carried out, with the result that, in many instances, what was thought to be a

protein monolayer actually was a monolayer in combination with adsorbed unspread protein. The apparent film thickness varied from anywhere between 10 and 45 Å. (discussed in reference 348). Also, the assumption that the thickness of a protein layer adsorbed on a metal slide equals that of the film on an aqueous surface often may be misleading (124).

The work of Rothen *et al.* (348) is free of such ambiguities. It was found that monolayers of metakentrin, a gonadotropic hormone of the anterior lobe of the pituitary gland, and monolayers of the oxytocic pressor hormone of the posterior lobe of the pituitary were practically inactive, in contrast to surface films of insulin which were as active as the native protein. While the specific intrinsic configuration of the molecules of the pituitary hormones is thus essential for their biological activity, this does not seem to be so with insulin, unless one assumes that surface denaturation of the latter is a reversible process. While the high cystine content of insulin may render its component polypeptide chains susceptible to reversible contraction, analogous to keratin fibres, there is no experimental evidence to sustain such a hypothesis.¹⁸ There is, however, evidence that in the denatured state proteins may exhibit certain manifestations of biological activity. For instance, monolayers of metakentrin, although devoid of hormonal activity, react specifically with antisera to the native protein (348). Monolayers of egg albumin, of serum proteins (348, 350), and of other antigenic materials (43, 92) likewise are capable of combining specifically with their homologous antisera (Part III E). *Vice versa*, denaturation of antibodies by various means (136, 350) does not impair the specific reactivity with the homologous native antigens. It appears, therefore, that certain kinds of biological activity require the specific configuration of the native protein, whereas others reside merely in the specific amino acid structure of the polypeptide chains, regardless of the latter's mode of folding.

E. IMMUNOLOGICAL PROPERTIES OF DENATURED PROTEINS

Attempts to elucidate the relation of protein structure to immunological properties have been most fruitful in those instances in which the protein antigen was chemically modified, thereby giving rise to a new serological specificity characteristic of the chemical constituents which had been introduced (218). Although it has long been known that treatment with typical denaturing agents may also alter profoundly the immunological behavior of antigens and antisera, only recently has significant progress been made in this field, largely owing to availability of pure material and improved experimental technique. Earlier observations on the effects of heat, acids, alkali, irradiation, and sound waves already have been summarized (69, 183, 253).

1. Surface denaturation

A direct approach to the problem under consideration lies in the study of the immunological properties of protein films, for if denaturation of globular

¹⁸ *In vivo* reversal of denaturation is not excluded by these experiments.

proteins is essentially a steric rearrangement of constituent groups in the molecule, then the complete spreading of a protein into a thin film represents an optimum degree of denaturation (see introduction). Such studies have been carried out with certain enzymes (151, 176, 220, 364) and hormones (348), as well as with a number of antigens and antibodies. In 1938 Shaffer and Dingle (359) showed that a film of egg albumin, 40 Å. thick and adsorbed on a stearate surface, could react with its antiserum. The activity of films of antibodies to types I, II, and III pneumococci, and of diphtheria toxin (33 Å.) was similarly demonstrated¹⁹ by Porter and Pappenheimer (328). However, since it has been established that a fully spread protein monolayer rarely exceeds 8–9 Å. in film thickness (293), the above investigations leave room for doubt, for such thick films may represent merely a layer of oriented, undenatured antibody molecules. Dean *et al.* (124) have emphasized the extreme care necessary to obtain monolayers uncontaminated by dissolved protein.

By an ingenious method, Chambers *et al.* (91, 92) found the two surfaces of presumably fully expanded protein films derived from hemolytic streptococci to behave differently in agglutination reactions, indicating the indifference of serological activity to specific configuration and the possibility that activity may reside in discrete structural features. Unfortunately, the film thickness in the former communication (91) was not specified, while in the latter (92) the films (14–20 Å.) represent at best only partially unfolded molecules.

Rothen and Landsteiner (349) clearly demonstrated the ability of a monolayer of egg albumin, 8–9 Å. thick, to combine with its antiserum. This investigation was extended (348) to insulin, to oxytocic pressor hormone, and to metakentrin, the gonadotropic hormone of the anterior lobe of the pituitary gland. More recently, Rothen and Landsteiner (350) studied the serological activity of films of egg albumin, horse and human serum albumins, and serum globulins; all were found to combine specifically with their antibodies. Likewise, films of purified anti-SSS I and anti-SSS III could specifically fix the corresponding polysaccharide, as evidenced by the deposition of another layer of antibody after treating a film with antigen solution.

2. Denaturation by urea and guanidine hydrochloride

Urea, guanidine hydrochloride, and other amides in high concentrations have been shown to be effective denaturing agents, causing partial unfolding of certain protein molecules (75, 295, 296, 300). Apparent reversal of this process resulted in a molecule of size, shape (295), and electrophoretic mobility (358) approximating those of the native material, whereas the specific internal configuration was not fully regained (64, 297). An investigation of the comparative antigenic behavior of the native and the modified proteins revealed (135) that although crystalline horse serum albumin regenerated from 8 *M* urea solution had lost over 90 per cent of its ability to elicit the formation of precipitins in rabbits, it had retained its original specificity and could combine with either antisera to the same titer as the native protein.

¹⁹ A finding not entirely in agreement with other observations (122).

Similar results were obtained with bovine serum albumin regenerated from urea or guanidine hydrochloride (254), although with this protein the more powerful denaturing action of guanidine hydrochloride was required to decrease significantly the antigenic activity of the naturally weakly antigenic, native protein. These investigations are of particular significance, not only since they were carried out with a pure protein, well defined in the native and the denatured states, but also as they demonstrate clearly the contribution of the intact internal configuration of this protein to antigenic activity.

Recently, it has been suggested that antibody globulin differs from normal globulin in the spatial configuration of the polypeptide chains rather than in chemical composition, and that denaturation of normal globulin, followed by regeneration in the presence of an antigen, should give rise to homologous antibodies (316). This hypothesis was supported by corresponding experiments in which the formation of a small amount of weak antibodies was observed (317). However, in keeping with this hypothesis, denaturation of an antibody or normal globulin, followed by regeneration in the absence of the homologous antigen, should give rise to a material essentially devoid of serological specificity; for, in the absence of specific directive forces, statistically only a small fraction of the molecules should revert to that configuration essential for activity. Such experiments were performed recently by some of the present authors, using purified antipneumococcal horse serum globulin type I and normal horse pseudoglobulin, denatured by and regenerated from 8 *M* guanidine hydrochloride (136).

It was found that the irreversibly denatured, and regenerated antibodies were strongly precipitated by the homologous antigen (SSS I), although in different optimum ratios than the native, while the native, irreversibly denatured, and regenerated normal globulins were all devoid of serological activity. These findings, to be discussed in detail elsewhere (137), suggest not only the independence of antibody activity to a specific structure, already demonstrated by surface-film studies, but also differences between normal and antibody globulin in chemical structure, i.e., the nature and sequence of amino acid residues in the polypeptide chains.

3. *Miscellaneous observations*

As early as 1896, Salter (351) observed a decrease in antigenicity of serum by heating. Since then, a number of reports have been published dealing with changes in the immunological properties of various antigens and antibodies upon treatment with a diversity of reagents (see 183, 218). Although there is general agreement that the altered protein antigens possess decreased antigenicity and a new specificity, and that the treated antisera react sluggishly, limited recognition of the nature of these denaturation processes renders an interpretation of these observations difficult. Nevertheless, several investigations are worth mentioning at this point.

(a) *Heat*: The effects of heat on the immunological properties of normal and immune sera, isolated antibodies, and a number of virus antigens have been

extensively investigated (45, 46, 47, 201-203). With all viruses studied,—i.e., tomato bushy stunt virus, tobacco mosaic virus, potato "X" virus, and tobacco necrosis virus,—infectious activity could be destroyed with little or no detectable changes in serological or physical properties. This was accomplished by mild heat treatment, irradiation with x-rays or ultraviolet light (48, 49), low concentrations of urea (52), or freezing under controlled conditions (54). Chemical agents, such as formaldehyde, nitrous acid, or hydrogen peroxide, produced similar effects (48-51, 370). More drastic treatments led to a higher degree of denaturation, as evidenced by a loss of serological activity accompanied by changes in chemical and physical properties.

Mixtures of proteins form complexes when heated to 75° to 80°C., as evidenced by salting-out (201) and electrophoretic studies (206, 397) (Part III C).

When somatic antigens are heated at 80°C. in the absence of non-specific protein and salts, they still can precipitate homologous antibodies; however, addition of non-specific proteins and salts before heating gives rise to complexes behaving like non-precipitating haptens (45).

Injection of non-precipitating complexes of both tomato bushy stunt virus and human serum globulin into rabbits gives rise to antibodies identical in behavior with those against unheated antigens (46). Similar heat experiments on antisera and isolated antibody globulin fractions showed a difference in behavior, depending on the type of homologous antigen used (202), i.e., rod-shaped or somatic. Rabbit antisera to the former lost all serological activity upon mild heating, whereas antibodies to the latter gave rise to non-precipitating complexes when heated with non-specific albumin. These complexes were capable of combining with antigen but incapable of neutralizing infectivity, specific precipitation, or complement fixation (47). While differences were found when heated in the presence of albumin, isolated antibodies to both types of antigens were equally susceptible to heat.

(b) *Irradiation*: In general, irradiation of proteins by ultraviolet light, alpha rays, or visible light in the presence of a photosensitizer leads to the formation of heterogeneous solutions (pages 164 and 220). It has long been known that, following irradiation with ultraviolet light, antigens react more weakly with antisera to the untreated protein (42). More recently it has been found that ultraviolet inactivation of urease renders the enzyme non-antigenic (325), while antibodies to irradiated tobacco mosaic virus still can neutralize the infectious activity of the virus (370). Progressive photooxidation of certain antisera causes a progressive lowering of the potency of these sera, with a simultaneous loss of tryptophan and histidine (363). Normal horse serum irradiated for several days with visible or ultraviolet light possesses a new specificity and a decreased antigenicity (183).

(c) *Acid and alkali*: Treatment of egg albumin with acid or alkali decreases the antigenic activity of the protein and alters its serological specificity (144, 197, 250, 405, 414). Acid treatment of pneumococcus rabbit antibodies lowers the ratio of mouse protection units to precipitable *N* and results in a decreased ability to fix complement, while the property of capsular swelling as well as the ability to transfer anaphylaxis passively are retained (402). The C'_2 and C'_4

components of complement have been shown to be unstable in acid but stable in alkali, the opposite being true for C'_1 and C'_3 (324). Preparation of apparently non-antigenic beef sera by treatment with alkali has been described (22, 132).

(d) *Enzymatic digestion*: Treatment with papain or pepsin under special conditions produces abrupt changes in the state of dispersion of egg albumin, diphtheria antitoxin, and beef globulins (4, 319, 320, 322, 392). The antigenic qualities of diphtheria antitoxin are greatly impaired upon digestion by pepsin (327, 403). Staphylococcus toxin, although completely detoxified by controlled peptic digestion, still possesses a marked antigenic capacity (314b). Under similar treatment, solutions of anti-SSS I horse globulins become partially non-precipitable by trichloroacetic acid, while the remaining protein can still combine with antigen, although in a ratio lower than that characteristic of the untreated protein (153, 321). More recently it has been reported that digestion by pepsin or diastase renders antitoxic plasma more resistant to subsequent heat treatment, decreases the size of the antitoxic molecule, and alters its antigenic character (198).

4. General considerations

While a widespread theoretical and practical interest has stimulated numerous investigations on the immunological properties of modified proteins, in only a few instances has such a degree of precision been attained as to permit interpretation of the data in terms of protein structure. Evidence derived from denaturation of well-defined proteins by surface forces or by urea or guanidine hydrochloride, suggests that the serological activity is independent of a specific internal configuration, while the antigenic activity appears to be impaired by processes leading to profound changes in protein structure. Beyond this, little or nothing is known about those attributes of a protein which endow it with immunological activity. There is no doubt that this aspect of the problem of protein denaturation requires vigorous investigation with the precision demanded by present-day standards of immunology and protein chemistry.

IV. IS DENATURATION REVERSIBLE?

The problem of the reversibility of denaturation occupies a key position in considerations of protein denaturation and protein structure. It centers around the question whether and to what extent a denatured protein can regain the specific chemical, physical, and biological properties characteristic of the native form. Experience has shown that certain proteins, such as serum albumin, hemoglobin, trypsin, or lactoglobulin (232), are capable of reverting from the denatured insoluble state to a soluble form which resembles the parent native protein in one or more properties. Other proteins, such as egg albumin, appear to be incapable of this conversion, or at least capable to only a limited degree.²⁰

²⁰ The observation that, following denaturation by urea or heat (75, 236, 406), a fraction of egg albumin fails to precipitate upon adjustment to the isoelectric point, suggests partial regeneration rather than incomplete denaturation of the protein.

For a given protein, the extent of "reversal" may vary with the conditions applied for denaturation, and, moreover, may depend on the procedure chosen for reversing the denaturation process. For instance, inactive denatured trypsin may be converted into a soluble active form when heat denaturation is carried out in acid, but not in alkaline, solutions (305). Rapid neutralization of acid-denatured hemoglobin yields practically no soluble protein (10), whereas more than 50 per cent may be rendered soluble upon slow neutralization (272).

The ultimate answer to the present problem lies in a precise comparative structural analysis of the native and the reversibly denatured protein, extending all the way down to the spatial arrangement of the constituent atoms and groups. As this is beyond experimental approach one has to rely on a study of measurable changes elicited on *denaturation*, with the object of ascertaining whether some or all of them can be *reversed*. *A priori*, such an analysis is not wholly conclusive, in that some irreversible changes in properties may occur which are not revealed by the particular properties open to investigation by present-day methods. On the other hand, any single irreversible change in property must constitute a sufficient criterion for the irreversibility of the denaturation process. The question can thus be answered negatively by proving the irreversibility of any single change, but not positively since it is impossible to demonstrate the identity of a multitude of properties of native and of reversibly denatured protein.

The mere fact that one or the other of the reactions accompanying denaturation can be reversed may be of considerable importance. Thus, the work of Northrop, Kunitz, and Herriott on the reversible inactivation of certain proteolytic enzymes (*vide infra*) has gone a long way toward identifying the enzyme proteins with the focus of biological activity, and has furnished valuable information on the nature of the inactivation process. Similarly, the apparently reversible changes in absorption spectrum of hemoglobin, following reversal of various kinds of denaturation to which the protein was subjected, have thrown light on the functional relation between protein carrier and prosthetic group. The nearly reversible changes in molecular asymmetry of serum proteins testify to the presence of forces which provide for the long-range elasticity of globular and fibrous proteins alike.

Just as the denaturation process has been studied by a limited number of methods, so has the reversal of denaturation. The most important of these are (1) solubility in salt solutions, (2) reactivity of chemical groups, (3) biological activity, (4) methods relating to molecular size and shapes, and (5) crystallinity.

1. Early observations

Michaelis and Rona (257) were probably the first to describe a reversal of protein coagulation. These authors observed that a coagulum of serum albumin, obtained by heating a solution at nearly neutral reaction and then adjusting the pH to the isoelectric point, could be redissolved in acids and remained soluble on readjustment to the isoelectric point. The protein now behaved like a "genuine protein", also in that it was again susceptible to heat coagulation. Spiegel-Adolf found that heat-coagulated serum albumin could be made to regain in part the solubility properties of the native protein by dispersing it in *N*/100

sodium hydroxide, followed by dialysis and electro dialysis (366). This "regenerated" protein resembled native serum albumin also in optical activity and serological properties. Anson and Mirsky (14) showed that if acidified solutions of serum albumin were heated and neutralized while the solutions were hot, complete precipitation of the protein ensued, while neutralization after cooling yielded about 70 per cent of soluble crystallizable protein. The same yield was obtained upon neutralization of solutions of acid acetone-precipitated serum albumin. Similar experiments were carried out by these authors with crystalline hemoglobin (10). In one (272) of a series of investigations (10-12, 272, 273) the heat-coagulated protein was dissolved in an excess of sodium hydroxide; after standing at room temperature, the solution was carefully neutralized with acid. With *oxidized ox* hemoglobin, very little soluble material could be recovered unless sodium hydrosulfite or sodium cyanide was added, whereas neither *oxidized* nor *reduced horse* hemoglobin was capable of regeneration unless cyanide was present, in which case 30 per cent of the protein became soluble. Native and regenerated hemoglobin were reported to resemble each other in crystallinity, color, and absorption spectra, and in being capable of reversible oxygenation (272). When denaturation was effected by hydrochloric acid, regeneration could be achieved by careful neutralization even without cyanide, yielding about 50 per cent of water-soluble hemoglobin (273). Similar procedures have been followed for the preparation of soluble globin from acid-coagulated globin (11, 13) with a yield of about 65 per cent for horse globin and 80 per cent for ox globin. The regenerated globins were capable of combining with heme to form compounds exhibiting the absorption spectrum of native hemoglobin, as discussed previously (page 216).

That reversal of the denaturation process may result in the reactivation of enzymes was demonstrated by the work of Northrop, Kunitz, and Herriott and of Anson and Mirsky on crystalline trypsin (17, 214, 305), chymotrypsin (215), and pepsinogen (185). Inactivation and denaturation were effected by heat or alkali, leading to a proportionate decrease in enzymatic activity and soluble protein. Following regeneration, the activity was regained in proportion to the amount of protein that became soluble. This parallelism between activity and protein solubility is significant, since later work has shown that some proteins may remain active under conditions which usually cause denaturation (235, 348), whereas others may regain their original solubility and other characteristic properties without being biologically active (53, 54).

Reversal of inactivation of insulin was observed by du Vigneaud *et al.* While heating at 100°C. in *N*/10 hydrochloric acid yielded a water-insoluble physiologically inactive precipitate, regeneration by alkali restored both biological activity and solubility in acids (130, 131). Partial reactivation has been found to occur also when insulin was inactivated by acid alcohol, yielding an easily hydrolyzable *N*-methyl compound (94), or when inactivated by 0.7 *M* ammonium hydroxide. Treatment with sodium or potassium hydroxide led to irreversible inactivation, while sodium carbonate or disodium phosphate was without effect (408).

In order to evaluate critically the experimental data pertaining to a reversal

of denaturation, we shall consider three proteins which have been most thoroughly studied with a combination of methods.

2. Serum albumin

When crystalline horse serum albumin is denatured by concentrated solutions of urea or guanidine hydrochloride, gross changes in molecular shape occur (page 222), though the molecular weight remains essentially constant (page 212). Removal of the denaturing agents by dialysis results in the separation of two fractions, one of them being insoluble in the pH region of the isoelectric point of the native protein, and the other remaining soluble (295).

At constant protein concentration, the amount of soluble "regenerated" serum albumin decreases in a sigmoidal manner with the concentration of denaturing agent originally present, reaching a lower limiting value of about 85 per cent of the total protein with concentrations of urea higher than 6 *M* and of guanidine hydrochloride higher than 4 *M*. With bovine albumin (331), the limiting yield of "regenerated" protein varies with the degree of "purity" of the preparation, being about 95 per cent for whole albumin containing 0.4 per cent of carbohydrate, in contrast to about 50 per cent for carbohydrate-free crystalline serum albumin. The extent of regeneration is thus seen to depend not only on the animal species but also on the method of preparation of the protein in question. Recent experiments (299) have shown that the quantitative distribution between insoluble "irreversibly" denatured protein and soluble regenerated protein depends also on the initial protein concentration, the yield of regenerated material increasing as the protein concentration is decreased. The parallelism between the relation of concentration of denaturing agent to increase in molecular asymmetry on the one hand, and to yield of "regenerated" protein on the other, is of significance. Since each concentration of urea, or of guanidine hydrochloride, produces a definite increase in the molecular asymmetry of each protein molecule, one may conclude that the greater the extent of change in molecular configuration, the smaller the fraction which returns to a soluble state. Since diffusion and viscosity measurements indicate that the regenerated protein resembles closely the native material in molecular size and shape properties, the regenerated protein may be associated with a more condensed configuration in contrast to the extended configuration of the asymmetric, denatured protein. It might be argued that incomplete reversal may have arisen from incomplete denaturation of the protein, the regenerated material corresponding to an undenatured fraction, or else from the presence of two fractions in the native protein, one of them being incapable of regeneration. The first of these hypotheses can be dismissed by the observed monodispersity of solutions of denatured serum albumin, as revealed by diffusion measurements, discussed in detail elsewhere (298). The second hypothesis is equally improbable, since denaturation of the regenerated fraction again gives rise to two fractions of soluble and insoluble protein, respectively, in the same distribution as was found following regeneration of the original denatured protein. The mechanism underlying this separation is not altogether clear. Comparison of the shape

properties of the denatured protein in the presence of the denaturing agent, and of irreversibly denatured serum albumin in buffer solutions, reveals the latter to be in a more condensed state than the former. Since, moreover, the relative dimensions of the denatured protein molecules are of considerably lower order of magnitude than those they would exhibit if they were fully extended polypeptide chains, it is apparent that they possess a high degree of internal cohesion which causes them to recoil when the denaturing agent is removed (297).

The suggestion has been made that incomplete regeneration was due to the interaction between denatured molecules, causing the latter to aggregate before they had an opportunity to recoil fully to the condensed configuration characteristic of the regenerated state (297). This hypothesis has received further support from the findings that the size of the fraction of regenerated protein increased upon decrease in protein concentration. However, since even in the more concentrated solutions (2 per cent) the mean interparticle distance exceeds by far the range over which common types of attractive forces are operative, the problem of the mechanism responsible for incomplete regeneration of this, as well as of other proteins that have been similarly studied (296), remains open.

The regenerated horse serum albumin resembles the native protein in molecular-kinetic properties, in crystallinity, and in serological specificity (135). However, it can be distinguished from the latter by three criteria: (1) It reveals a higher electrophoretic mobility on the alkaline side of the isoelectric point (358) and shows a greater amount of electrophoretic boundary spread. (2) It is more susceptible to tryptic fission, being hydrolyzed at a rate higher than that of the native protein and the same as that of the irreversibly denatured material (64, 331). (3) Although it gives rise to the same type of antibodies as the native protein, its capacity to produce antibodies is greatly impaired (135, 254). On the strength of one or all of these criteria, denaturation of serum albumin has to be considered as an irreversible process.

As discussed previously (page 222) it is not clear whether heat denaturation of serum albumin causes the same type of changes in the protein molecule as does denaturation by urea, guanidine hydrochloride, or synthetic detergents. Though at the temperature of heating the protein becomes insoluble at the isoelectric point (14, 366), the solubility is regained in part if the heated solutions are readjusted to the pH of the isoelectric point after cooling (340). Though the material is devoid of reactive disulfide groups, it can be crystallized and is serologically equivalent to the native protein (259). Its molecular-kinetic properties are different, at least under the specific conditions at which the latter were determined (118). In the absence of quantitative measurements, it is not possible to decide whether the incomplete regeneration following denaturation by heat, or by acid acetone (14), is due to the same factors previously considered for the regeneration of the urea-denatured protein.

3. Hemoglobin

The denaturation of the hemoglobins by urea, acid, heat, and sodium salicylate has been considered earlier in this review. The present discussion is concerned

primarily with the reversal of these processes and with the question of the identity of regenerated hemoglobin with the native protein. Specifically, the regeneration following denaturation by (a) urea, (b) acid, and (c) sodium salicylate will be considered together with the analogous reactions of the globin moiety.

(a) *Denaturation by urea*: Evidence previously considered (page 217) suggests that the following reaction occurs when carboxyhemoglobin (horse) is exposed to concentrated urea solutions for limited periods of time:



where H_4 denotes heme and Gl denotes globin. Upon prolonged contact of the reactants denaturation is supposed to proceed further, much in the same manner as denaturation by sodium hydroxide (128), yielding a compound of oxidized heme and denatured globin; this, in turn, is split into alkaline hematin and denatured globin. However, the latter reactions occur very slowly, and considerably more slowly with carboxyhemoglobin than with oxyhemoglobin.

Reaction 1 can be reversed in part by removal of urea or similar denaturing agents (formamide and acetamide) by dialysis (374). As in the case of serum albumin, the amount of regenerated protein decreases with increasing concentrations of the denaturing agent and, in analogy with serum globulin (296), it varies also with the nature of the denaturing agent. Thus, according to Steinhardt (374), with 4 *M* urea 70 per cent of the protein becomes soluble, with 6.5 *M* urea less than 50 per cent, and with 7.5 *M* urea practically none. With formamide, the total protein can be recovered in a soluble, regenerated form.²¹ The distribution is independent of the rate or other conditions of dialysis.

It is difficult to decide whether reaction 1 is reversible for that fraction that reverts to a soluble state. As has been shown in Parts II B and III A, denaturation by urea proceeds essentially along the same path as the first step of the denaturation by acids, i.e., a dissociation of the molecule into halves, initiated by the rupture of two hydrogen bonds. If bond formation is restricted to two special groupings, then recombination would likewise be restricted to these very groupings and denaturation would be a truly reversible process. However, the fact that half-molecules are formed with both types of denaturation does not necessarily indicate that they are limited to the same mechanism. Moreover, the observations that the regenerated protein shows a greater amount of boundary spread in sedimentation, and a greater tendency for aggregation, are suggestive of the existence of some differences between native and regenerated protein (374).

(b) *Denaturation by acid*: This reaction occurs in two stages, as discussed previously, i.e., dissociation according to reaction 1, followed by



²¹ Indicating again the inadequacy of a single criterion for estimating the extent of denaturation. Thus, any one of these amides produced the same change in molecular weight; yet the degree of regeneration is quite different.

Reaction 1 occurs between pH 6 and pH 4, while reaction 2 begins at pH 3.9 and is probably complete at pH 3.1 (190). According to a preliminary note by Drabkins, reaction 2 is a slow one (128), whereas Mirsky and Anson observed that 3 min. after exposure to dilute hydrochloric acid, all the protein was precipitated upon rapid neutralization with sodium hydroxide at 0°C. (273).

The yield of soluble regenerated hemoglobin, obtained after careful neutralization with sodium hydroxide in the presence of sodium cyanide, depends on the time of contact with cyanide and increases from 10 per cent after 1 min. to about 75 per cent after 65 hr. (273). In the absence of cyanide, about 50 per cent of the protein is obtained in soluble form. Denatured globin, prepared by acetone precipitation from a solution of acid-denatured hemoglobin, likewise yields about 70 per cent of soluble regenerated globin (13).

The question as to whether reaction 2, i.e., the conversion of native to denatured globin, is strictly reversible cannot be answered at the present time, since globin has yet to be isolated without the intermediary step of denaturation. However, if it is tentatively assumed that reaction 1 is reversible, then full identity of native and regenerated hemoglobin would prove the reversibility of reaction 2. Mirsky and Anson reported that regeneration of heat-coagulated hemoglobin yields a material which is indistinguishable from the native protein in crystal form, coagulation temperature, color, position of absorption bands, and ability to combine reversibly with oxygen and carbon monoxide (272). Holden (190, 191), in a series of investigations, demonstrated the identity between native and acid-regenerated carbon monoxide hemoglobin with respect to their characteristic absorption bands. As noted previously (page 216) the regenerated protein is devoid of free sulfhydryl groups, in contrast to the denatured protein, and exhibits the same molecular size and shape properties as the native protein (339). However, differences were observed (1) in susceptibility to dissociation by alkali, (2) in electrophoretic mobility, and (3) in the shape of the oxygen dissociation curve (see page 216). It is difficult to decide whether these changes, testifying to the lack of identity between native and regenerated hemoglobin, should be ascribed to the irreversible character of reaction 1 or 2. However, as the latter gives rise to more drastic changes in the protein molecule, i.e., splitting away of the heme moiety accompanied by denaturation of the globin part (as witnessed by the exposure of sulfhydryl groups, and insolubility of the protein), it is apparent that reaction 2 would be less likely to be reversible than reaction 1. We are inclined, therefore, to blame the irreversible nature of the over-all reactions mainly on the failure of denatured globin to revert to the characteristic configuration of the original protein, in analogy with the regeneration of serum albumin, although it remains to be established whether under conditions favoring reaction 1, at the exclusion of reaction 2, dissociation of hemoglobin into half-molecules can occur in a truly reversible fashion.²²

(c) *Denaturation by sodium salicylate*: The reaction between hemoglobin and sodium salicylate has been cited by Anson and Mirsky (18) in support of the

²² See, however, the lack of identity of native and recombined *Helix pomatia* hemocyanin, as revealed by electrophoresis (393).

existence of mobile equilibrium between native and denatured protein, which is progressively shifted toward the denatured side as the concentration of salicylate is increased.

According to this theory, denaturation is an all-or-none reaction in that the protein is in either a native or a completely denatured state, the concentration of the denaturing agent merely governing the quantitative distribution between these two forms. This concept, based upon limited observations, is incompatible with subsequent evidence derived from investigation of the effects of increasing concentrations of urea and other denaturing agents on proteins (160-162, 290, 295-297), according to which an increase in the concentration of a denaturing agent results in a *uniform* increase in the *degree* of denaturation. For this reason it is necessary to examine more closely the mechanism of the interaction between hemoglobin and sodium salicylate. Salicylate-denatured hemoglobin differs from the native protein in solubility, absorption spectrum, and susceptibility to tryptic digestion (18).

For measuring the postulated equilibrium between native and denatured protein, advantage was taken (18) of only one of these properties, i.e., an almost twofold increase in the absorption of green light on the part of the denatured hemoglobin, as compared to the native.²³ However, the intensity of light absorption varied with time, increasing at first, followed by a stationary level only to increase again. The mere fact that the same stationary level was established when the "equilibrium" mixture was made by starting with native or with denatured protein, is by itself not unequivocal proof for the existence of a state of equilibrium, particularly since aggregation of the denatured molecules has been found to have an influence on the degree of light absorption. Moreover, recent work has shown that colorimetric measurements alone are unreliable for determining the extent of the reaction between hemoglobin and sodium salicylate (336).

While the spectra of methemoglobin and of carboxyhemoglobin are quite different, the spectrum of methemoglobin in the presence of salicylate resembles that of carboxyhemoglobin alone, probably owing to complex formation between salicylate and the iron of methemoglobin. Though salicylate has little effect on the spectrum of carboxyhemoglobin, the heat of this reaction is about the same as that accompanying the interaction between salicylate and methemoglobin. On the other hand, while sodium benzoate changes the methemoglobin spectrum in a similar manner as does sodium salicylate, the heat of this reaction is practically zero. Therefore, not only are changes in spectrum unspecific expressions of the denaturation process, but the reaction between salicylate and hemoglobin is far from stoichiometric, since the heat of the reaction continues to increase above salicylate concentrations at which, according to Anson and Mirsky (18), the reaction has gone to completion (336).

The qualitative changes observed for the interaction between salicylate and hemoglobin have been found to be reversible. The regenerated protein is also indistinguishable from the native material in osmotic pressure (338, 341) and in

²³ Actually 1.7 fold (238).

susceptibility to splitting by alkali (339), in contrast to preparations regenerated after denaturation by heat or acid. It has been suggested that the combination between the protein and the salt is a loose though specific one, conceivably owing to the tendency of the salicylate molecule toward hydrogen-bond formation (336).

4. *Trypsin*

The fundamental studies of Northrop, Kunitz, and Herriott on the reversible inactivation of crystalline trypsin (214, 305), chymotrypsin (215), and pepsinogen (185) have so much in common that they may be adequately covered by a more detailed discussion of but one of these, i.e., trypsin.

(a) *Heat*: Crystalline trypsin may be reversibly inactivated by heat (305) in the pH range from 1 to 7, whereas in more alkaline solutions the reaction is irreversible. That at higher temperatures the enzyme is inactivated may be demonstrated by the fact that upon rapid addition of cold salt solution complete precipitation occurs, the filtrate being wholly inactive. In these, as in the following experiments, heating was confined to a short period, since prolonged exposure to high temperatures always resulted in irreversible inactivation. When salt was added after the protein solutions were allowed to cool, no precipitation occurred, leading to a complete restoration of the initial solubility and enzymatic activity. The equilibrium between active native and inactive denatured protein is rapidly established, and is governed by the temperature of heating. For instance, at 20°C. the protein is fully active and soluble, whereas upon heating at 40°C. about 50 per cent of the protein becomes precipitated by one-fourth saturation with ammonium sulfate, accompanied by a proportionate loss in activity. The same quantitative distribution is obtained by cooling solutions to 40°C. (17).

The rate of inactivation increases with increasing temperature, the reaction being nearly instantaneous at 90°C. Reactivation, however, is a comparatively slow process, requiring about 10 min. for completion when the protein in *N*/20 hydrochloric acid is rapidly heated to boiling and then poured into a mixture of *N*/20 hydrochloric acid and cracked ice.

At pH 7, the relation between inactivation and denaturation is more complex. When the protein is heated to temperatures ranging from 40° to 60°C., the amount capable of reversible inactivation and denaturation decreases in exactly the same manner as the amount which is inactivated and denatured. In this temperature range, denaturation and inactivation are irreversible processes following the general pattern of the heat inactivation of biological materials. However, above 60°C., total activity and total protein, i.e., the amounts that can be recovered following reversal of denaturation, increase with increasing temperature, approaching a value of 100 per cent above 90°C., whereas above 70°C. no residual protein or activity could be detected prior to reversal of denaturation. These observations have been interpreted by Northrop and Kunitz in terms of two competing reactions: (1) an equilibrium between active native and inactive denatured trypsin, which is shifted toward the right as the temperature is raised. Accordingly, the amounts of residual soluble activity

and protein decrease in proportion until all the protein is in the inactive denatured state, above 60°C. While subsequent to acidification and cooling, the enzyme tends to revert wholly to the native active state, this reaction is inhibited by another reaction, i.e., (2) the digestion of inactive denatured trypsin by the active native form. No reactivation occurs in the temperature range in which inactivation proceeds at a measurable rate, i.e., 40–60°C., since hydrolysis of the inactive denatured trypsin causes a progressive shift in the equilibrium of the first reaction toward the denatured form. At higher temperatures, however, the inactivation is practically instantaneous and complete, and no active trypsin remains, thereby permitting a reversal of the denaturation process proper.

This hypothesis offers an explanation for most of the observed effects, although it fails to explain why in the higher temperature range of inactivation the activity is restored to a smaller extent than the solubility. For instance, after heating at 70°C., over 80 per cent of the total protein becomes soluble in contrast to 30 per cent of the activity, the corresponding values being (by extrapolation of Northrop's data (305)) 90 and 50 per cent for 80°C. and 80 and 100 per cent for 92°C.

(b) *Alkali*: Exposure of solutions of active native trypsin to alkali results in rapid *irreversible* inactivation, the course of the reaction depending on the pH of the solutions (214). However, when the solutions are titrated in the cold to increasingly alkaline reactions, and then *instantly* added to cold 0.01 *M* hydrochloric acid, the inactive trypsin reverts within several hours to the native active state. This *reversibly* inactivated protein remains soluble upon the addition of *M*/1 sodium chloride, while precipitation ensues if salt is added immediately after acidification of the alkali-denatured protein. Thus, as in the case of heat inactivation, denaturation by alkali is a rapid process, while the reactivation proceeds considerably more slowly. The extent of inactivation increases with increasing pH, being complete above pH 13. Under the conditions just described, reactivation occurs in proportion to the amount of protein nitrogen that becomes soluble.

The rate of *irreversible* inactivation, occurring upon prolonged contact with alkali, depends on the pH and the temperature. For instance, while at pH 13 about 25 per cent of the enzyme may still be reactivated after 5 min. standing at 0°C., at 30°C. complete and irreversible inactivation occurs instantaneously. While upon prolonged standing the total amount of protein decreases, probably owing to slow hydrolysis, this does not occur within the period of time requisite for complete inactivation. Hence, the lack of reactivation at pH 13, as well as at pH 2, cannot be ascribed to a digestion of denatured trypsin by residual active enzyme. Within the pH range of 2.5 to 10, inactivation is also a rapid process. However, here the irreversibility of the process appears to be due to the slow digestion of the inactive denatured protein by the native active protein, analogous to the process described for the irreversible inactivation by heat.

The situation may be summed up by stating that after short contact with alkali, denatured trypsin slowly regains, on addition of acid, the activity and

solubility properties of the native enzyme, whereas longer exposure to alkali leads to permanent inactivation and denaturation.

5. General considerations

The preceding discussion has shown that under suitable conditions several denatured proteins may regain certain properties characteristic of the native state. The most tangible, though by no means most decisive, property common to native and regenerated protein is solubility in water or in salt solutions, and on the basis of this criterion the extent of denaturation varies greatly with the nature of the protein and of the denaturing agent. For instance, with horse serum albumin denatured by urea or guanidine hydrochloride 85 per cent of the protein has been recovered in a soluble regenerated state (295). Under similar conditions, 35 per cent of regenerated horse serum globulin has been obtained following denaturation by 8 *M* urea, and 15 per cent following denaturation by guanidine hydrochloride (296). With urea-denatured egg albumin less than 15 per cent of soluble protein is obtained (74), whereas urea-denatured myosin or tobacco mosaic virus protein appear to be wholly incapable of regeneration (133, 168, 381). Similar considerations apply when biological activity is used as a criterion for estimating the degree of regeneration. Thus, under special conditions trypsin, chymotrypsin, and pepsinogen may be completely reactivated, inactivation being otherwise partly or wholly irreversible. Inactivation of pepsin is always largely irreversible (303), while crystalline papain does not become inactivated for appreciable periods of time even in the presence of concentrated urea solutions (235).

In order to view the problem of reversible denaturation in the proper perspective, it is necessary to consider the nature and magnitude of the changes which a protein undergoes under the influence of a given denaturing agent, and to inquire about the reliability of specific criteria as valid indicators for the absolute identity of native and regenerated protein.

Urea denaturation of several proteins, e.g., serum albumin, serum globulin, egg albumin, and myogen, has been pictured as a transition from a specific condensed to a less specific extended configuration, caused by a partial unfolding of polypeptide chains. This interpretation appears to be fully sustained by experimental evidence. Acid denaturation of hemoglobin has been shown to be initiated by a dissociation of the molecules into halves, followed by a cleavage of the subunits into heme and denatured globin. The common feature of all these processes is a profound change in the physical structure of the protein molecule.

The first requisite for strict reversal of denaturation is a long-range elasticity of the polypeptide chains to facilitate their contraction to the condensed configuration; the second, that there be a mechanism which directs each residue and group to the same steric position which it occupied in the native protein molecule.

Evidence previously considered testifies to the contractile properties inherent in fibrous proteins (page 234) and in denatured globular proteins (page 236) alike. The question of the nature and site of the attractive forces responsible

for contraction is a matter of conjecture but is of profound importance in considerations of the second requisite for truly reversible denaturation.

On purely statistical grounds it would be extremely unlikely that each and every residue and group will find its way back to the specific relative position which it held in the native state, unless one or the other of the following hypotheses be evoked:

(1) The state of folding of polypeptide chains is determined primarily by attractive forces acting between constituent groups of the main chains. Hence, as long as these forces remain active, a partially unfolded polypeptide chain could revert to the original condensed configuration. This hypothesis implies that the nature and sequence of constituent amino acid side chains are relatively unimportant as structure-determining elements, and, since the main chains have the same atomic arrangement in all proteins, that proteins have a common and unique intrinsic structure. This hypothesis appears to be discredited by the noted influence of side-chain composition on the structure and contractility of protein fibres (see Part III D) and by the observed variations of globular proteins in susceptibility to denaturation and regeneration.

(2) Side-chain interaction being accepted as a predominant factor in molding polypeptide chains to the condensed configuration specific for individual proteins (30, 76), strict reversal of denaturation would be conceivable if, for some reason, an unfolded chain is forced back into the unique configuration of the native protein. This singleness of structure could be due either to the predominance of specific attractive forces or else to the steric requirements of the side chains, permitting of only one single state of folding.

Specific attractive forces may be found in cross linkages of the disulfide type. Indeed, the position of cystine residues is fixed in a polypeptide chain and it has already been shown that disulfide bridges confer on proteins a high degree of contractility (Part III D). However, though a cursory inspection of analytical data reveals that many proteins which are capable of regeneration have a notably high cystine content, others which are poor in, or even devoid of cystine, also exhibit a specificity of structure. Hence, it is unlikely that such covalent cross linkages are exclusive structure-determining factors. Salt linkages and hydrogen bonds between side chains undoubtedly are equally important as structure-determining elements. However, the great number of side chains capable of forming bonds of that type would permit of a variety of combinations once a polypeptide chain has become unfolded by denaturation. Therefore, the concept of side-chain interaction alone may fail to account for a singleness of structure.

It has been shown that the space requirements of amino acid residues, notably of those containing aromatic or heterocyclic rings, greatly restrict the internal rotation and steric freedom of polypeptide chains (288) and that the presence of glycine residues tends to alleviate these restrictions (291). However, analytical data reveal that with most proteins amino acids of smaller cross-sectional areas predominate, thereby allowing sufficient freedom for a chain to assume various configurations (*cf.* 55). While a more precise consideration of this problem is

unwarranted at the present time, we are inclined to accept the view that genuine polypeptide chains may fold up in a number of ways and that upon refolding, the mode of refolding will be governed to an appreciable extent by merely statistical factors, thus rendering the specific configuration of the native protein one of several equally probable ones (297). Thus, the mode of refolding but not refolding itself limits the true reversal of the denaturation process.

The opening up in protein structure concurrent to denaturation is preceded by an "activation" of the native protein molecule. The nature of this activation process is not generally understood though in two instances, i.e., pepsin and hemoglobin, it has been identified with the ionization of specific groups (Part II B). While chemical reactions of that or similar types are thus precursors of changes in physical configuration, there is no reason to believe that the former may not occur without being always followed by the latter. This, obviously, will depend on whether such groups occupy key positions in the structure of the molecule.

That chemical reactions with protein surface groups may go a long way in profoundly altering characteristic chemical and biological properties of native proteins is well known. Thus, the activity of enzymes (*viz.*, trypsin, pepsin, urease) and the solubility of proteins are highly sensitive to subtle changes in the degree of ionization as is the susceptibility of proteins to denaturation. Such purely chemical reactions may very conceivably be reversible, even if they should lead to intermolecular aggregation, provided the physical structure of the protein remains intact after redispersion of the aggregates. In short, if denaturation were to be confined to reactions of and between amino acid side chains, without significantly altering the internal configuration of the protein, such processes may suffice to influence appreciably certain characteristic properties of the native protein and may be reversible, in contrast to any process which affects the state of folding of the polypeptide chains themselves.

It is premature to decide whether such speculations have sufficient reality to account for the apparent reversal of denaturation of trypsin by heat or alkali or of hemoglobin by sodium salicylate. However, it is a matter of fact that the inactivation of trypsin is a rapid process, while reactivation occurs slowly and only under the influence of acids. If inactivation were to entail a dissociation of carboxyl groups, as it does in the case of pepsin (page 201), possibly followed by aggregation, reactivation would consist in disaggregation, usually a slow process, followed by reversal of the initial step. Under the prolonged influence of heat or alkali, interaction might proceed so far as to prevent depolymerization without causing more profound and permanent intramolecular changes. The solubility behavior of heat-denatured serum albumin or hemoglobin might be explained by similar reasoning. Thus, neutralization of the hot solutions causes precipitation of the polymerized denatured molecules; when neutralization is preceded by cooling, the depolymerized protein remains soluble subsequent to neutralization. The permanent changes in the state of aggregation occurring upon prolonged heating have been evinced by molecular-kinetic analysis (118).

Mirsky and Pauling's argument that heat-inactivated trypsin is distinguished

from the native protein by a considerably higher entropy content (278) does not invalidate the present conclusions. Calorimetric studies on the inactivation of pepsin have shown that the over-all heat of the reaction is probably that of several chemical processes, one of them being the inactivation proper (116). Indeed, ΔH , and the activity of this enzyme change in a different manner with pH, the latter decreasing less rapidly than the former. Hence, by analogy, the entropy of inactivation of trypsin is probably less than that considered in Mirsky and Pauling's calculation, which already has been shown to be of doubtful significance (page 208). Moreover, as discussed elsewhere (297), for a protein molecule comprising several thousands of bonds, such a change in entropy may be readily accounted for by an infinitesimal change in vibrational motion, thus leaving the inactive enzyme in a practically equally specified state as the native. The amount of opening up in protein structure, therefore, may be negligible.

Several proteolytic enzymes appear to be remarkably resistant to usually powerful denaturing agents. Tryptic activity is measured in the presence of concentrated urea solutions (52), pepsin remains active for several hours in 4 *M* urea (374), and papain retains its activity in 9 *M* urea for as long as 24 hr. (235). Since heat denaturation frequently produces less profound changes than does denaturation by urea and similar amides, it appears that the inactivation of these enzymes by heat is associated with other and probably milder changes than those experienced by the action of the aforementioned organic reagents.

On the basis of these considerations, the biological activity of certain proteolytic enzymes seems to be dependent not only on the intact physical configuration of the protein but also on the presence of specific "active" groups. This is in contrast to other manifestations of biological activity which, as discussed previously (page 239), are rather insensitive to gross changes of chemical or physical nature.

In summary, it may be said that the more profound the changes in a protein which occur upon denaturation, the less likely is denaturation to be reversible. While in many instances the regenerated protein has regained many of the characteristic properties of the native state, the concept of denaturation as a reversible reaction does not warrant general application.

Finally, it should be stressed that these considerations apply to the reversal of the *in vitro* denaturation of proteins, while little, if anything, is known about its occurrence *in vivo*. It is apparent that at some stage in the synthesis of proteins polypeptide chains fold up in order to be molded into a specific configuration, under the controlling influence of intracellular enzymes (60). Conversely, unfolding will occur prior to the hydrolytic action of extracellular proteinases. While the synthesis and degradation of proteins are continuous dynamic processes, it is idle to speculate whether these reversible processes ever halt at the stage of denaturation. Several specific phenomena have been postulated to involve reversible denaturation. One of these, i.e., the reversible contraction of muscle, has been referred to (Part III D). Others are the effect of temperature on the reversible quenching of bacterial luminescence (196) and the reversible conversion of visual purple to visual yellow (264).

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CIS-TRANS ISOMERIZATION AND STEREOCHEMISTRY OF CAROTENOIDS AND DIPHENYLPOLYENES

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I. INTRODUCTION

The literature concerned with the stereochemistry of unsaturated carbon compounds has increased considerably in volume during the last few decades.

However, successful investigations which have demonstrated the presence of a large number of *cis-trans* isomers of a given compound are scarcely known.

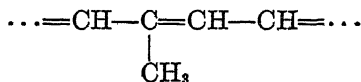
Simple and effective methods were needed which would enable the experimenter to differentiate and to purify a multitude of stereoisomers. Such methods can be efficient only if each isomer shows some degree of stability and yet a marked tendency for further isomerization. It is improbable that procedures will be found for the direct synthesis of each stereoisomer. The preparative approach will lie rather in the conversion, under carefully defined conditions, of an easily available or naturally occurring isomer into many others.

In this review we intend to present a survey of some recent progress in the stereochemistry of polyenes.

Compounds which possess a large number of double bonds have been named "polyenes," a term which tacitly implies the aliphatic character and the conjugated position of these bonds. The presence of such a long conjugated system is made evident by the color. The diphenylpolyenes, $C_6H_5(CH=CH)_n C_6H_5$, are the simplest synthetic compounds of this type which have been thoroughly studied (see p. 335), but the vegetable and animal kingdoms offer a much greater variety in the form of the carotene-like pigments, the so-called "carotenoids."

A. THE NATURE OF CAROTENOIDS

In this class are found yellow, orange, red, and violet pigments which are free of nitrogen and which may or may not contain oxygen. All carotenoids are built up from isoprene units; the most common ones possess eight such units and, consequently, forty carbon atoms. Those isoprene groups which are located in the central part of the molecule are dehydrogenated thus,



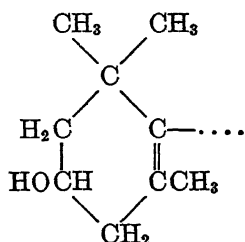
and form part of the chromophore, whereas those near or at the ends of the molecule are hydrogenated.

Carotenoids are insoluble in water but soluble in fats or in fat solvents. A first characterization of a carotenoid pigment can be achieved by the well-known "partition test." If a petroleum ether or benzene solution is shaken with 85 per cent methanol, hydrocarbon pigments are found mainly or entirely in the upper layer ("epiphasic behavior"); in contrast, xanthophylls, i.e., carotenoid alcohols containing at least two free hydroxyl groups, transfer into the lower layer ("hypophasic behavior"). Fully esterified xanthophylls behave like hydrocarbons, whereas monohydroxy carotenoids are epiphasic with respect to 85 per cent methanol but hypophasic (or mainly so) with respect to 95 per cent methanol.

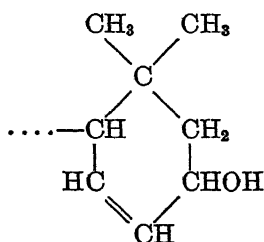
Carotenoids may be hydrocarbons, alcohols, aldehydes, ketones, or acids. α -Carotene, β -carotene, γ -carotene, and lycopene, all of which have the formula $C_{40}H_{56}$, are the most important hydrocarbon carotenoids. The most widespread carotenoid alcohols in nature are the following: cryptoxanthin (a monohydroxy-

β -carotene), $C_{40}H_{55}OH$; zeaxanthin (a dihydroxy- β -carotene), $HOC_{40}H_{54}OH$; and lutein ("xanthophyll", a dihydroxy- α -carotene), $HOC_{40}H_{54}OH$. In some carotenoids, ketone groups occur: for example, in rhodoxanthin, $C_{40}H_{50}O_2$, and capsanthin, $C_{40}H_{58}O_3$. The few carotenoids with acid character are of lower molecular weight and are probably formed by oxidative cleavage of a C_{40} -pigment in the vegetable tissue. Bixin ($CH_3OCCC_{22}H_{26}COOH$), crocetin ($HOOC-C_{18}H_{22}COOH$), and azafrin ($(HO)_2C_{26}H_{35}COOH$) belong to this subclass (p. 332).

No carotenoid has yet been found which has an oxygen-containing group in the middle section of the molecule. Hydroxyl or carbonyl groups are usually in a position meta to the quaternary carbon atom in the hydroaromatic ring. The following formulas and those on p. 276 illustrate the main structural features mentioned. For details a monograph may be consulted (102).



Two terminal groups
in zeaxanthin; one
such group is present
in cryptoxanthin



One terminal group
in lutein; the other
is identical with
the formula at the left

B. HISTORY AND METHODS OF *cis-trans* ISOMERIZATION IN THE FIELD OF THE CAROTENOIDS

1. History

So far as is known, the discovery of a second form of bixin by Herzig and Faltis (17) in 1923 was the earliest experimental contribution to the stereochemistry of polyenes. Their observations were interpreted as *cis-trans* rearrangement by Karrer and his associates six years later (21).

In the field of the C_{40} -carotenoids the pioneer observation was made by Gillam and El Ridi (9) in 1935. These investigators reported that if homogeneous β -carotene is washed from an alumina column with petroleum ether (or eluted with methanol) and then re-adsorbed, it separates clearly into two zones which show the spectral bands of β - and α -carotene, respectively. "The lower zone thus contains a pigment formed during the adsorption process, and moreover elutriation and re-adsorption of either of the two zones gives another separation into two zones with properties as before, on each subsequent adsorption, the process thus being reversible and never complete." The new zone, "pseudo- α -carotene," according to Gillam, cannot be identical with α -carotene because

the latter on similar treatment yields a pigment with maxima at shorter instead of longer wave lengths.

In two subsequent papers (10, 11) in 1936-37 Gillam and his associates made a closer study of the phenomenon and confirmed the complete reversibility of the process



As an explanation of these phenomena, both double-bond migration and geometrical isomerization were discussed by the British authors.

The cause of the isomerization was attributed to the action of the adsorbent, that is, to the chromatographic process itself. This interpretation, however, had to be abandoned. It was observed in 1937 by Cholnoky and the author (104) that capsanthin solutions undergo a spontaneous reversible isomerization; furthermore, nearly simultaneous investigations of lycopene, β -carotene, and cryptoxanthin in collaboration with Tuzson showed that the phenomena are independent of the adsorption process (127, 129). In favorable cases the isomerization can be followed by optical readings without the use of a Tswett column (128).

The reversible changes are spontaneous and occur with much greater rapidity at higher temperatures: for example, when a solution is refluxed. In 1938 Strain (82) reported that heat alters some xanthophyll pigments, and that these then show a complex chromatogram and modified spectral curves. The classic catalyst of *cis-trans* changes, iodine, used previously in the field of bixin and crocetin (p. 322), was introduced into the stereochemistry of C_{40} -carotenoids by Tuzson and the author in 1939 (128). For further historical data *cf.* Jones (18).

Although in our first investigations other processes, e.g., a keto-enol tautomerism in capsanthin, had to be taken into consideration, as the work progressed it was possible to state with increasing certainty (105, 106, 117, 128) that the conversions observed must generally be classified as *cis-trans* rearrangements (*cf.* also the discussions given by Jones (18) and Hunter *et al.* (16)). When the great number of double bonds in a polyene molecule is considered, it becomes evident that a difficult stereochemical problem, which requires special methods, has presented itself.

2. Methods

A long conjugated double-bond system offers many spatial possibilities, but the synthetic as well as the natural polyenes are usually all-*trans* compounds. The following sections of this review will show, however, that this configuration can be easily altered by various thermal, photochemical, or catalytic treatments. Upon such treatment the stereochemical uniformity of the molecules disappears and a complicated mixture of *cis-trans* isomers is formed, in which ordinarily the unchanged portion of the initial all-*trans* pigment still predominates. Theoretically, each possible stereoisomer must be present in such an equilibrium mixture even if only in minute quantity.

The chemical characteristics of the stereoisomers of a given carotenoid are very similar, but fortunately the adsorption affinities are highly dependent on configuration, perhaps more so than any other known physical property. Therefore, the Tswett method of chromatographic adsorption (85, 103) is the only effective technic for the separation and study of the stereoisomers. After suitable development with a solvent, the pigment adsorbed on a vertical column separates into the individual stereoisomers (or groups of them), which appear in distinct zones. Each zone may be cut out, eluted, transferred into another solvent, and examined as desired. The properties, including the readiness, direction, and rate of further *cis-trans* changes, may be studied. Some of the *cis* compounds crystallize easily, while others are labile and can be investigated only in solution.

The chromatographic procedure is also frequently used in order to decide whether or not two pigments from different sources are identical. A mixture of the two compounds is chromatographed: a single zone indicates identity, but two zones point to dissimilarity. This method of the "mixed chromatogram" is very useful in stereochemical work, especially because only minute quantities of material are required for the test.

Spectroscopic methods play an important part in stereochemical studies, as the spectra are strongly influenced by spatial features. The nature of an isomer may be determined rapidly by inspecting the bands in a visual spectroscope before and after the addition of a catalytic amount of iodine, which converts the solute into a stereoisomeric equilibrium mixture. More precise information can be obtained by a study of extinction curves. Stereochemical alteration finds its specific expression not only in the visible region but more particularly in a certain part of the ultraviolet.

It is to be hoped that the combined application of isomerization methods, chromatography, and spectroscopy will stimulate further advance in this field and will finally enable the chemist to deduce the configurations of many *cis-trans* isomers.

After some necessary suggestions concerning nomenclature we shall discuss in more detail the stereochemistry and *cis-trans* isomerization of the C₄₀-carotenoids in Part II, of the carotenoids of lower molecular weight in Part III, and finally, of the diphenylpolyenes in Part IV. In none of these sections has the literature been completely covered, because of present circumstances.

C. NOMENCLATURE

The term "stereoisomeric set" includes all *cis-trans* isomers of a given carotenoid and each stereoisomer is a "member" of the set.

If a pigment is designated by its current name, for example, " β -carotene", this refers usually to the all-*trans* form, in which each double bond possesses *trans* configuration. The prefix "all-*cis*," however, has a more restricted sense. It denotes a compound in which all double bonds which are spatially unhindered in assuming the *cis* configuration are present in the *cis* form. As will be explained later, some double bonds must necessarily remain *trans* because of spatial con-

flicts. Consequently, an "all-*cis*" pigment still retains some *trans* double bonds. Between these limits lies a great number of partially-*cis* isomers for which a special nomenclature is necessary.

We propose the following system (120): Each double bond of the chromophore will be assigned a number which will be italicized in order to avoid confusion with the numbering of carbon atoms: for example, *3,β-di-cis-β-carotene*. The lowest number will be given to the double bond in or nearest a *β*-ionone ring. In the absence of such a cyclic system, an *α*-ionone ring receives preference over an aliphatic terminal group. Conjugated double bonds in or near such open groups will be assigned the highest numbers. The numbering of entirely aliphatic and unsymmetrical molecules requires special decisions.

Because the configurations of isomers are still unknown in most instances, a temporary nomenclature is in use. It is customary to employ the prefix "neo": for example, neo-*β*-carotene A. The letters immediately following A in the alphabet designate isomers with weaker adsorbability than A; neo-*β*-carotene B is adsorbed below neo A. If a pigment (e.g., *α*- or *β*-carotene or cryptoxanthin) also yields isomers which adsorb above the all-*trans* form, those with increased affinity are designated as neo T, U, V, etc. The stereoisomers of zeaxanthin, lutein, and capsanthin which are adsorbed above the all-*trans* pigments are designated with the first letters of the alphabet. There is some inconsistency in this nomenclature, which arose on a historical basis.

The use of the prefix "pro" will be discussed later.

II. CAROTENOIDS CONTAINING FORTY CARBON ATOMS

A. CONFIGURATION OF PLANT CAROTENOIDS IN SITU

It can be reliably stated that the overwhelming quantity of the carotenoid pigments present in vegetable tissue possesses an all-*trans* configuration. This follows not only from the results of x-ray investigations of crystals (Hengstenberg and Kuhn (15); Mackinney (49)) and from the interpretation of spectra by Mulliken (56, 57, 58) and by Pauling (62), but also from the chromatographic examination of numerous extracts which have been prepared from various plant organs. The adsorption column usually does not show any appreciable quantity of *cis* compounds in fresh extracts, and even if such zones occur, the possibility of spontaneous isomerization during the extraction and subsequent operations must be excluded before the occurrence of *cis*-compounds in the tissue can be definitely asserted.

The abundant occurrence of the all-*trans* pigments in plants is understandable because they, of all possible spatial structures, possess the lowest energy content and the greatest stability.

The very prevalence of the all-*trans* form in nature stimulated efforts to find exceptions. The exclusive occurrence of a uniform configuration of carotenoids in the vegetable kingdom did not seem probable *a priori*. The first observations in this field, however, were not associated with C₄₀-carotenoids but with the lower-molecular-weight polyenes, bixin and crocetin (p. 322). The simul-

taneous occurrence of partially-*cis*- and all-*trans*-crocetins, which are present as gentiobiosides in the saffron stigma, is remarkable. The *cis*-isomer may easily be converted into the all-*trans* compound by iodine catalysis or irradiation. In discussing these rearrangements with reference to the biosynthesis of carotenoids, Kuhn and Winterstein (39) wrote: "The possibility must be considered that the stigma of saffron will yield substantially more *cis*-pigment if worked up in the completely fresh state with exclusion of light, heat and catalysts. A photochemical rearrangement of the pigment glucoside perhaps takes place already in the living plant when the flowers open. It will be especially important to test whether other carotene pigments, in particular the precursors of vitamin A, are primarily formed by the plants as labile geometrical isomers of the well-known compounds."

These predictions are in harmony with Schroeder's recent observation (72) that, if stems with the buds of "Monkey flowers" (*Mimulus longiflorus*, Grant) are placed in water, and the flowers allowed to open in diffuse light, substantial amounts of the *cis* compounds, prolycopene and pro- γ -carotene (see below), are present. In contrast, the pigment of the flowers developed on the intact plant in the open contains only the corresponding all-*trans* forms, *viz.*, lycopene and γ -carotene.

It has also been observed that some flowers show a yellow color if there is very little sunshine during their developmental period; during prolonged clear weather, however, the usual orange color appears. Such changes are still open to investigation. Evidently, the configuration of carotenoids *in situ* constitutes a factor which has so far been neglected and which is able to influence both intensity and shade of pigmentation.

That sunshine is necessary for the biosynthesis of some chromophoric structures was demonstrated long ago in the case of chlorophyll. The conjugated double-bond system of carotenoids, however, can be built up in darkness, as evidenced by the formation of pigment in carrots. Sunshine may nevertheless constitute an important factor in directing the initial spatial configuration into its final shape.

The chemical and stereochemical aspects of the carotenoid formation in *Cryptogames* are less well known. For example, van Deventer (4) claimed that light is necessary for the biosynthesis of lycopene and other carotenoids occurring in *Neurospora sitophila*. According to Lederer (43) the pigmentation of a red yeast, *Torula rubra*, is much strengthened by diffuse light. Other cases probably could be found in the literature.

That *cis* structures can be protected and preserved in the vegetable tissue even in prolonged and intense sunshine, is shown by the occurrence of the so-called pro-carotenoids in fruits; the same pigments are stereochemically sensitive when insolated in solutions.

Two representatives of this stereochemically new type of C_{40} -carotenoids, prolycopene, $C_{40}H_{56}$, and pro- γ -carotene, $C_{40}H_{56}$, have recently been found in higher plants. The first qualitative observation was made in collaboration with LeRosen, Went, and Pauling (117), and the isolation of pure crystalline samples

was carried out by LeRosen (45), Schroeder (122, 123), Escue (109), and the author. Whereas the positions of corresponding visible spectral maxima of the bixin and crocetin *cis-trans* pairs differ in wave length by a few millimicrons only, this difference (in petroleum ether) attains 35 $m\mu$ and 31 $m\mu$ for prolycopene-all-*trans*-lycopene and pro- γ -carotene-all-*trans*- γ -carotene, respectively. As will be shown below, probably six *cis* double bonds are present in prolycopene and five in pro- γ -carotene. These pigments possess characteristically flat extinction curves which undergo spectacular changes upon a photochemical or catalytic treatment.

TABLE 1
Occurrence of prolycopene and pro- γ -carotene in some plants

FAMILY	PLANT	ORGAN	STATE OF DRYNESS	CRYSTALS ISOLATED FROM 1 KG.	REFERENCE
Prolycopene					
<i>Solanaceae</i>	<i>Lycopersicum esculentum</i> (Mill.), var. Tangerine tomato	Fruit pulp	Fresh	mg. 20	(45)
<i>Palmae</i>	<i>Butia capitata</i> (Becc.)	Fruit pulp	Fresh		(121)
<i>Palmae</i>	<i>B. eriospatha</i> (Becc.)	Fruit pulp	Fresh		(121)
<i>Pomoideae</i>	<i>Pyracantha angustifolia</i> (Schneid.)	Whole fruit	Air-dried	28.4	(123)
<i>Celastraceae</i>	<i>Evonymus fortunei</i> (L.)	Seeds	Air-dried	11	(109)
<i>Scrophulariaceae</i> ..	<i>Mimulus longiflorus</i> (Grant)	Petals	Fresh		(124)
Pro- γ -carotene					
<i>Palmae</i>	<i>Butia capitata</i> (Becc.)	Fruit pulp	Fresh	0.3	(122)
<i>Palmae</i>	<i>B. eriospatha</i> (Becc.)	Fruit pulp	Fresh		(122)
<i>Pomoideae</i>	<i>Pyracantha angustifolia</i> (Schneid.)	Whole fruit	Air-dried	27.7	(123)
<i>Celastraceae</i>	<i>Evonymus fortunei</i> (L.)	Seeds	Air-dried	0.5	(109)
<i>Scrophulariaceae</i> ..	<i>Mimulus longiflorus</i> (Grant)	Petals	Fresh		(124)

Representatives of this poly-*cis* class of carotenoids seem to be widespread in nature, as evidenced by the fact that the six plants listed in table 1 belong to five families.

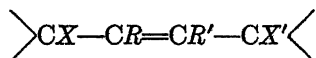
B. THE POSSIBILITY OF STERIC CHANGES IN POLYENIC STRUCTURES

Although the spatial possibilities offered by a long conjugated system are manifold, they have been studied only recently. In 1904 Werner wrote in his textbook (93) that he did not know any compound in which more than two double bonds capable of *cis-trans* changes were present. As late as 1931 Wittig and Wiemer (101) stated that when the number of conjugated double bonds

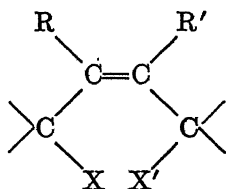
increases, the phenomenon of *cis-trans* isomerism is pushed to the background and finally disappears, because of the increased mobility of the valence electrons (cf. 3, 100). Two years later, however, Kuhn (26) correctly summarized the situation by the following statement: "... according to the available evidence a strong accumulation of double bonds does not exclude the occurrence of *cis-trans* isomerism. That the higher diphenylpolyenes are known only in one spatial form is due to the inadequacy of the preparative methods." This is in accordance with Ebel's statement (6) that the non-existence of many isomers foreseen by theory is caused by their rapid rates of rearrangement.

It can be reliably stated on the basis of recent experimental evidence, that the length of an aliphatic double-bond system does not affect its ability to assume various *cis-trans* configurations; in some carotenoid sets a dozen stereoisomers have been observed.

We shall now proceed to a discussion of the number of theoretically possible *cis-trans* isomers of a given conjugated system. First of all, it must be stressed that not all double bonds of the system are necessarily available for a spatial rearrangement. They can be divided into "stereochemically effective" and "stereochemically ineffective" double bonds, the latter being hindered by some spatial conflict in assuming *cis* configuration. In a C_{40} -carotenoid, the chromophore of which is composed of dehydrogenated isoprene groups, only one double bond in each C_5 unit of the aliphatic chain is able to assume *cis* configuration. According to Pauling (62), these are those double bonds which carry methyl side chains, and in addition the central double bond; the latter has a privileged structural and stereochemical position (see the formulas on page 276). The stereochemically ineffective conjugated double bonds are those which are located in a ring or which are adjacent to a $C-CH_3$ group. The latter type of double bond is ineffective because of the steric interaction between the hydrogen of CH and the methyl of $C-CH_3$. Thus, in a chain with the structure



the *cis* form will be oriented in the following way:



If X and X' are both hydrogen atoms, they will be 1.7 Å. apart. This is somewhat less than the usual distance of van der Waals contact, but the strain can be removed in large measure by small rotations out of coplanarity. If, however, X = methyl (or some similar group) and X' = H, the distance is only 1.6 Å. (figure 1), i.e., only half the distance of the van der Waals contact. The *cis* form would, therefore, certainly be unstable. Hence Pauling (62) concluded that in

the open chain of carotenoids, *cis* configuration will be assumed only by those double bonds which are of the type >CH-CR=CR'-CH< , that is, which are adjoined by two CH groups.

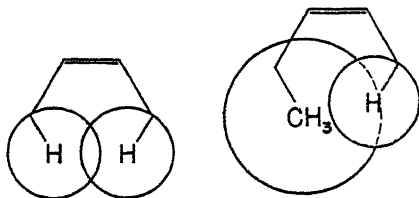
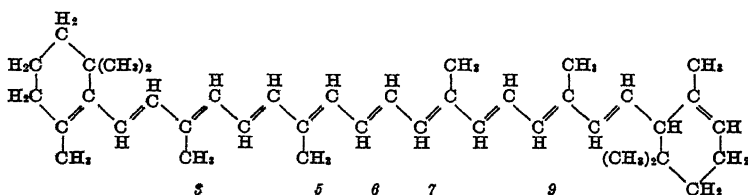
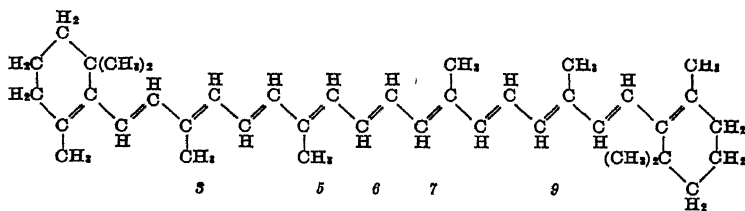


FIG. 1. Overlapping of hydrogen atoms in —CH—CR=CR—CH— and of hydrogen and methyl in —CH—CR=CR—CCH_3 with *cis* configuration.

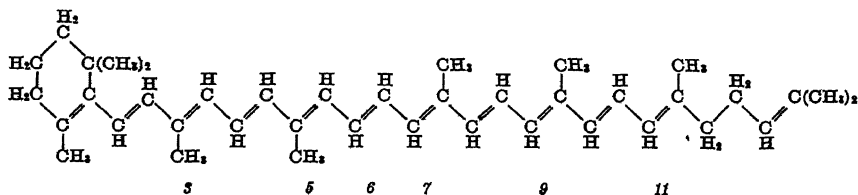
On the basis of these considerations the number of possible *cis-trans* isomers is much less in the highly branched carotenoid molecule than in an unbranched aliphatic compound with an equal number of conjugated double bonds. The positions of the stereochemically effective double bonds in the four important natural polyene hydrocarbons, the carotenes and lycopene, are indicated in the formulas below (the stereochemically effective double bonds are numbered):



All-*trans*- α -carotene
(The five stereochemically effective double bonds are numbered)



All-*trans*- β -carotene
(The five stereochemically effective double bonds are numbered)



All-*trans*- γ -carotene
(The six stereochemically effective double bonds are numbered)

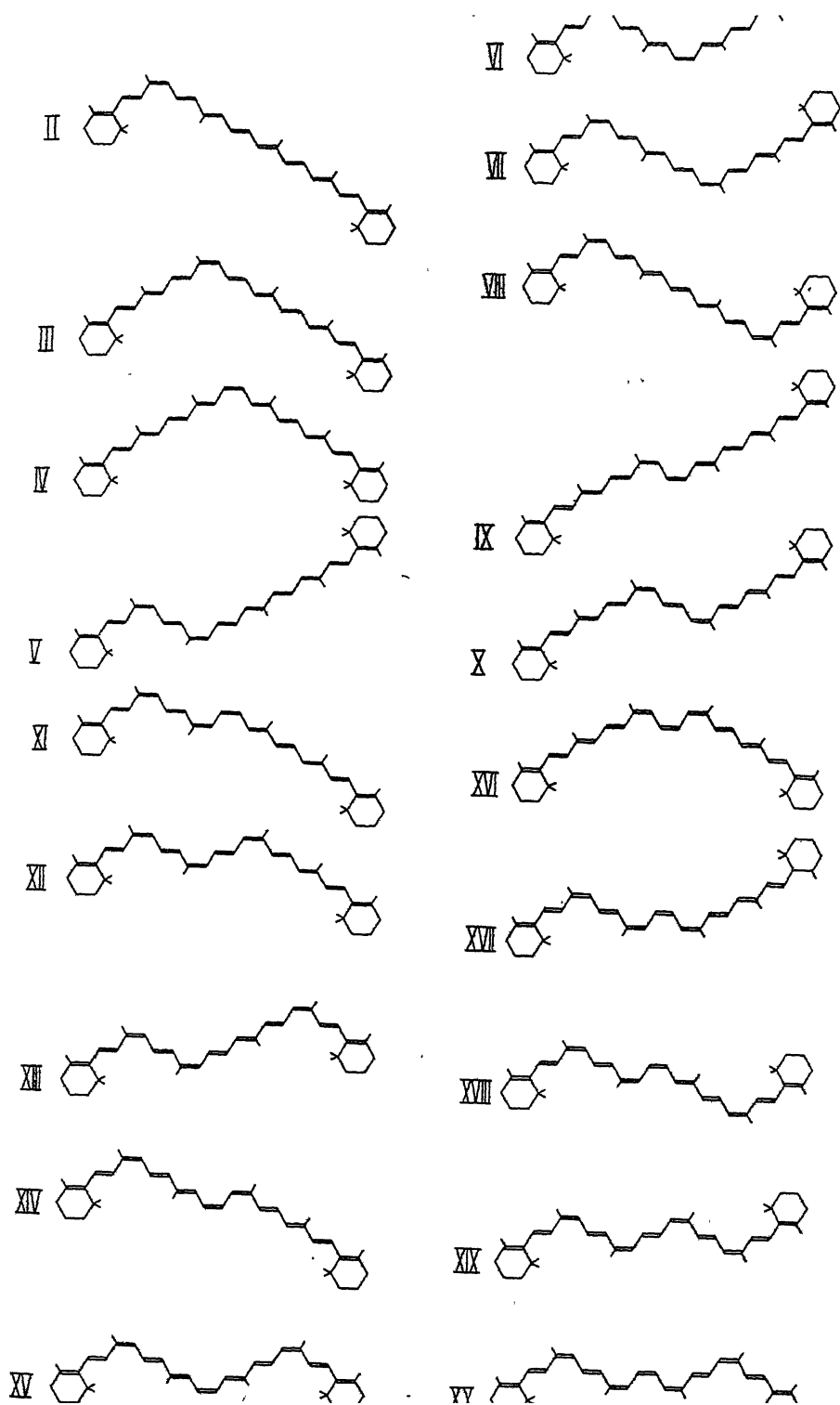
which can be utilized for the partial stereoisomerization of an all-*trans* compound or any of its isomers. Although the existence of such spatial changes may be

TABLE 3

Calculated number of stereoisomers for naturally occurring carotenoids and for some of their conversion products

PIGMENT	SYMMETRICAL (s) OR UNSYMMETRICAL (u) MOLECULE	NUMBER OF STEREO- CHEMICALLY EFFECTIVE DOUBLE BONDS	CALCULATED NUMBER OF STEREISOMERS
α -Carotene	u	5	32
β -Carotene	s	5	20
γ -Carotene	u	6	64
Lycopene	s	7	72
Cryptoxanthin	u	5	32
Rubixanthin	u	6	64
Gazaniaxanthin	u	6	64
Lycioxanthin	u	7	128
Lutein	u	5	32
Zeaxanthin	s	5	20
Physalien	s	5	20
Lycophyll	s	7	72
Rhodoviolascins	u	7	128
Rhodoxanthin	s	5	20
Capsanthin	u	5	32
Capsorubin	s	5	20
Astacin	s	5	20
Astaxanthin	s	5	20
Semi- β -carotenone	u	5	32
β -Carotenone	s	5	20
α -Citaurin	u	5	32
β -Citaurin	u	5	32
Methylbixin	s	5	20
Bixin	u	5	32
Norbixin	s	5	20
Crocetin	s	5	20
Methylcrocetin	u	5	32
Dimethylcrocetin	s	5	20
Azafrin	u	4	10
Vitamin A	u	2	4

demonstrated by optical methods (for example, spectroscopy, colorimetry, polarimetry, etc.), chromatographic analysis is the only means available for the separation of the individual isomers.



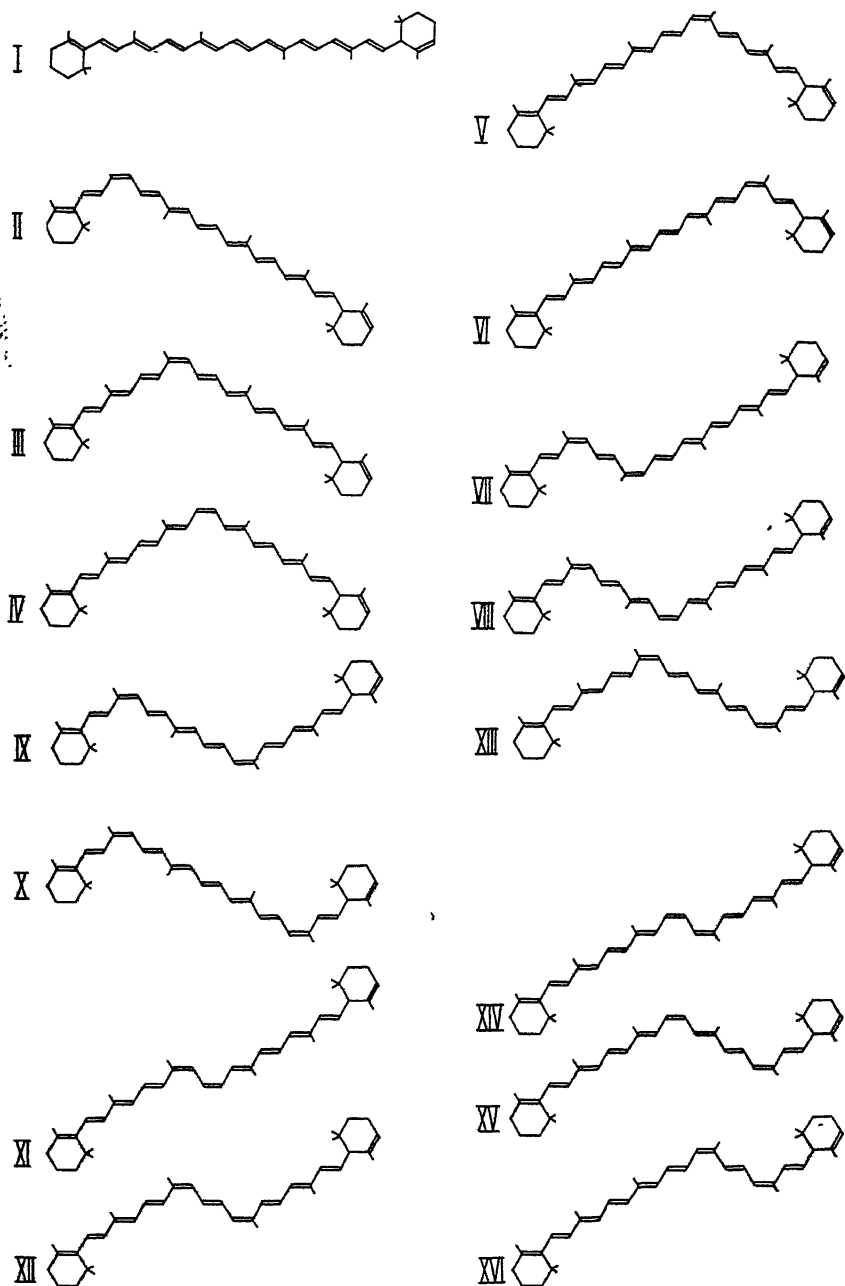


FIG. 3. Skeleton models of sixteen of the possible thirty-two stereoisomers of α -carotene: all-*trans*- α -carotene, five mono-*cis*- α -carotenes, and ten di-*cis*- α -carotenes.

No pigment zone observed on the Tswett column after an isomerization experiment should, however, be accepted without further criticism as containing

a member of the initial stereochemical set, because the possibility of a slight destruction or oxidation by air exists. Such processes can even become predominant under drastic conditions. Products of moderate oxidation are adsorbed near the top of the column, whereas cleavage products possessing a considerably shortened chromophore may pass into the chromatographic filtrate, to which they impart a pale color or a fluorescence when illuminated with ultraviolet light. In most cases the product of these unwelcome processes can easily be differentiated from stereoisomerized pigments: the isomerization is reversible, in contrast to oxidation and cleavage. When any stereoisomer is submitted to the treatment by which it was formed (or to an equivalent treatment), members of the same stereochemical set must appear.

This isomerization can be rapidly achieved by iodine catalysis. Because the all-*trans* isomer thus formed is present in preponderant or at least substantial amount, it can easily be separated on a new column and compared in a mixed chromatogram with an all-*trans* sample from another source. Pigment zones formed by current methods of isomerization which do not respond to a reversibility test represent losses in stereochemical work.

The following methods will now be considered somewhat more in detail: (1) thermal isomerization in solution; (2) thermal isomerization achieved by melting crystals; (3) isomerization induced by iodine catalysis or (4) by acid catalysis; and finally (5) photo-isomerization.

1. Thermal *cis-trans* isomerization in solution

To this category belong spontaneous isomerization at room temperature and isomerization on heating or refluxing in the absence of light or catalysts.

The phenomenon of spontaneous isomerization, which starts immediately on solution of crystals and proceeds much more rapidly upon refluxing, was observed with lycopene, β -carotene, and cryptoxanthin in collaboration with Tuzson (129). The changes which lutein or zeaxanthin solutions undergo on heating were described by Strain (81, 82, 83) and also by Tuzson (128) and Cholnoky, Polgár, and the author (106, 107). While several of Strain's observations were the first in this field, the reversibility of the process was not claimed in this early work nor was the action of heat clearly differentiated from an alleged effect of the column which had been advanced by Gillam and El Ridi (10).

The spontaneous isomerization of all-*trans* carotenoids at room temperature is a slow process; its rate depends on the solvent and on the pigment structure. We found, for example, that the following percentages of the starting material had isomerized in benzene or petroleum ether solution within a day: α -carotene, β -carotene, cryptoxanthin, and capsanthin, 1 to 2 per cent; gazaniaxanthin and zeaxanthin, 4 to 5 per cent; capsorubin, 8 per cent; lycopene, 10 per cent. According to Carter and Gillam (2), if β -carotene solutions are stored at -2°C . in the dark and protected from air, less than 3 to 4 per cent will undergo stereoisomerization in three months. Even partially-*cis* isomers may be preserved under similar conditions; thus, certain β -carotene isomers showed only 5 to 6 per cent re-isomerization after two months. The influence of the temperature on the stability of dissolved all-*trans*- β -carotene is illustrated in table 4.

When a dilute benzene or petroleum ether (b.p. 60–80°C.) solution of an all-*trans* carotenoid is refluxed, the equilibrium (or steady-state) mixture is reached within 15 to 60 min. The extent of this isomerization for certain carotenoids is summarized in table 5. The behavior of partially-*cis* compounds may vary within broad limits. Many such isomers have conversion rates which are similar to those of the all-*trans* form. Polycopene and pro- γ -carotene are, however, so thermostable that their extinction curves remain practically unaltered when the solutions in petroleum ether are refluxed for 30 min. (116).

Comparative data for five members of the β -carotene set (65) are given in table 6.

The rate of isomerization of β -carotene is higher in the non-polar solvent toluene, than in the polar solvent nitromethane (16).

TABLE 4

Percentage of β -carotene stereoisomers with decreased adsorption affinity formed at various temperatures in petroleum ether-benzene solution (Carter and Gillam (2))

TEMPERATURE	DURATION OF THE EXPERIMENT	ISOMERIZED FRACTION
°C.		<i>per cent</i>
20	1 day	1
	7 days	5.5
	49 days	11.1
40	1 hr.	4.0
	3 hr.	5.4
	24 hr.	11.2
60	1 hr.	7.5
	3 hr.	9.7
80	1 hr.	8.5
	3 hr.	31.9
	24 hr.	34.1

2. Cis-trans isomerization by melting crystals

If crystals of a carotenoid are kept a few degrees above their melting point, both reversible and irreversible changes occur. The conversion becomes manifest not only by a loss in the initial color intensity, which may decrease by more than half, but also by the appearance of fluorescing material in the filtrate of a chromatogram. The extent of cleavage is less if the pigment possesses a low melting point, if the duration of the experiment is only 1–5 min., and if the fusion temperature is depressed by addition of naphthalene. In no case do true equilibrium mixtures seem to be formed.

In practice the pigment is melted in a sealed tube filled with carbon dioxide. Then the melt is solidified by rapid cooling, dissolved, and chromatographed. Whether or not a zone contains a member of the initial set can be established by spectroscopy, by the partition test, or by mixed chromatograms.

The data in table 7 show that the all-*trans* form is by no means present in greatest quantity in the stereoisomeric mixture obtained, the composition of

TABLE 5
Cis-trans isomerization of all-trans carotenoids caused by refluxing solutions

PIGMENT	SOLVENT	DURATION OF REFLUXING	RATIO OF STEREOISOMERS (PER CENT OF PIGMENT RECOVERED)	REFERENCES
		<i>minutes</i>		
α -Carotene.....	Petroleum ether (b.p. 60–70°C.)	30	All- <i>trans</i> :neo U:neo B = 92:4:4	(120)
β -Carotene... ..	Petroleum ether (b.p. 60–70°C.)	60	All- <i>trans</i> :neo U:neo B:neo E:labile isomer = 86:4:8:1:1	(65)
Lycopene.....	Petroleum ether (b.p. 70–80°C.)	30	All- <i>trans</i> :neo forms = 45:55	(129)
Cryptoxanthin.....	Ligroin (b.p. 120°C.)	60	All- <i>trans</i> :neo A:neo B = 62:32:6	(113)
Gazaniaxanthin.....	Benzene	30	All- <i>trans</i> :neo forms = 70:30	(125)
Lutein... ..	Benzene	30	All- <i>trans</i> :neo forms = 89:11	(128)
Zeaxanthin	Benzene	30	All- <i>trans</i> :neo A:neo B = 70:24:6	(106)
Physalien.....	Petroleum ether (b.p. 70–80°C.)	60	All- <i>trans</i> :neo forms = 58:42	(106)
Taraxanthin.....	Benzene	30	All- <i>trans</i> :neo forms = 85:15	(128)
Capsanthin.....	Benzene	30	All- <i>trans</i> :neo forms = 80:20	(105)
Capsanthin dipalmitate.	Petroleum ether (b.p. 70–80°C.)	30	All- <i>trans</i> :neo forms = 64:36	(105)
Capsorubin.....	Benzene	30	All- <i>trans</i> :neo forms = 80:20	(105)

TABLE 6
Relative colorimetric values of β -carotene and some of its stereoisomers formed by refluxing for 60 min. in petroleum ether (b.p. 60–70°C.)

STARTING MATERIAL	RELATIVE PHOTOMETRIC VALUES (PER CENT)				
	Neo U	All- <i>trans</i>	Neo B	Neo E	Labile isomer
Neo- β -carotene U	31	40	19	10	
All- <i>trans</i> - β -carotene	4	86	8	1	1
Neo- β -carotene B	4	50	40	3	3
Neo- β -carotene E	14	49	19	18	-
Labile isomer	10	22	24	30	14

which is, of course, dependent on the conditions of the experiment. Some partially-*cis* pigments are only slightly less stable in the melt than the all-*trans*

form; for example, after having been kept in the melt at 115°C. for 5 min., more than half of polycopene was unchanged. The isomerized portion contained partially-*cis* members of the set and some all-*cis*-lycopene but only a trace of the all-*trans* form (45).

3. *Cis-trans* isomerization by iodine catalysis at room temperature

It is well known that iodine is a powerful catalyst which strongly influences the spatial structure of ethylene derivatives. In the field of C₄₀-carotenoids

TABLE 7
Stereoisomers formed by melting crystals of carotenoids

STARTING MATERIAL	TEMPERATURE	DURATION OF FUSION	RATIO OF STEREOISOMERS	REFERENCES
	°C.	minutes		
All- <i>trans</i> -α-carotene	195-200	15	All- <i>trans</i> :neo U:neo V:neo W:neo X:neo B:neo D = 35:12:6:19:7:14:7	(120)
Neo-α-carotene U.....	74	15	All- <i>trans</i> (with A):neo U:neo X:neo B = 23.5:66:7:3.5	(120)
All- <i>trans</i> -β-carotene	190	15	All- <i>trans</i> :neo U:neo V:neo A:neo B:neo E:labile isomer = 33:19:4:8:24:8:4	(65)
Neo-β-carotene U.....	135	15	All- <i>trans</i> :neo U:seven minor isomers = 22:40:38	(65)
All- <i>trans</i> -cryptoxanthin...	170	2	Some neo A, no neo U, mostly unchanged all- <i>trans</i>	(113)
	170	10	All- <i>trans</i> :neo U:neo A = 48:22:30	(113)
	170	15	All- <i>trans</i> :neo U:neo A:neo B = 49:7:38:6	(113)
All- <i>trans</i> -cryptoxanthin (with naphthalene)	115	5	Mostly unchanged all- <i>trans</i> , some neo A, less neo B, no neo U	(113)
All- <i>trans</i> -zeaxanthin (with naphthalene)	160	15	All- <i>trans</i> :neo A:neo B:neo C = 56:17:17:10	(113)
All- <i>trans</i> -capsanthin	180	1	All- <i>trans</i> :neo forms A + B + C = 29:71	(67)

it was first used in collaboration with Tuzson (128) when lycopene, β-carotene, lutein, cryptoxanthin, zeaxanthin, and taraxanthin were investigated. The earliest detailed studies referred to zeaxanthin and its ester physalien (with Cholnoky and Polgár (106)), and to capsanthin, capsorubin, and their palmitates (with Cholnoky (105)).

If iodine is added to a solution of an all-*trans* carotenoid, in quantities of 1 to 2 per cent of the pigment, rapid steric changes take place at room temperature. This leads, in most cases, to an equilibrium mixture in which all members of the

stereoisomeric set should theoretically be present. In practice, however, subsequent chromatography shows that only a limited number of stereoisomers, two to twelve, can be detected on the usual scale of laboratory work. Those members of the set which contain many *cis* bonds and which, therefore, are spectroscopically very different from the all-*trans* form, are not formed in detectable quantities.

The composition of the iodine equilibrium mixture is usually different from that of the equilibrium mixture obtained by the refluxing of solutions. This difference lies not only in the ratio in which the members of the set are present but even in the presence or absence of detectable amounts of certain isomers. For example, only a little neolutein B is obtained when a solution of all-*trans*-lutein is refluxed, but this isomer appears in substantial quantities upon iodine catalysis in the cold (128). The method of isomerization also influences the ratio of neozeaxanthins A and B and of neocapsanthins A and B (105). A limiting case is given by polycopene and pro- γ -carotene, which are only slightly isomerized by refluxing but in contrast are extremely sensitive to iodine. When they are treated with this catalyst, no detectable amounts of the starting material are present in the equilibrium mixture.

The outstanding feature of the stereochemical rearrangement which takes place in the presence of iodine is the reversibility of the process. If an isomer is separated on a Tswett column, eluted, transferred into a suitable solvent, and catalyzed again, all the pigments observed in the first chromatogram appear in a subsequent chromatogram. Theory requires that each member of a set give the same equilibrium mixture.

If the extinction values for such a mixture have been established by adding the catalyst to a solution of a weighed quantity of a crystalline member of the set, the concentration of any isomer belonging to the set, whether crystallizable or not, can be rapidly established. Some factors to be discussed later, however, may make such estimations inaccurate to the extent of ± 0.5 to 5 per cent.

In order to obtain information on the rates of isomerization, spectrophotometric readings can be made at regular intervals of time at a definite wave length until constancy (or quasi-constancy) has been reached. A sample of the solution may then be chromatographed and the ratio of unchanged to isomerized pigment may be determined.

Exact measurement of the kinetics of iodine catalysis is not yet available. The time needed to achieve steric equilibrium evidently is dependent on the concentrations and temperature. For practical purposes it can be stated that if the concentration of the pigment is of the order of magnitude of 0.1 mg. per milliliter of petroleum ether or benzene (1/5000 molar), and if the weight of the iodine is 1 to 3 per cent of the pigment, then at 25°C. equilibrium is reached within 15 to 60 min. (in many cases within a few minutes).

The dependence of the rate of isomerization on the quantity of catalyst is illustrated in table 8 (45).

As in many other cases of iodine catalysis, the stereoisomerization of carotenoids is promoted by light. One can even claim that for all practical purposes daylight or illumination with a lamp is necessary, although over-exposure may

cause destruction. Individual carotenoids behave very differently; lycopene is exceptionally sensitive. However, the behavior of lycopene will be considered and illustrated later in the discussion of the so-called "cis-peak" effect (p. 302).

Iodine is not only a catalyst for stereoisomerization but may also cause the formation of faintly colored cleavage products. This side reaction is shown through losses in the total color intensity which are established by a photometric balance after catalysis, chromatography, and recovery. In many cases this loss is negligible, but with polycopene (45) it amounted to 30 per cent in some experiments, although it was much less in others. In contrast, 97 per cent of the initial pigment was recovered in a blank determination in which no iodine was added.

Strain (84) observed that iodine catalysis of zeaxanthin produced not only the well-known stereoisomers, but also some pigments which were usually formed by acid catalysis. If the iodine solution was mixed with a little pyridine, quinoline, or dimethylaniline, the by-products did not appear. This result would possibly indicate the formation of traces of acid under the influence of iodine.

TABLE 8

Influence of the quantity of iodine on the rate of stereoisomerization of polycopene (45)

RATIO OF GRAM-ATOMS OF IODINE TO GRAM-MOLECULES OF PIGMENT	AMOUNT OF IODINE (PER CENT OF THE PIGMENT)	EXTENT OF ISOMERIZATION AT 25°C. WITHIN 1 MIN. (PER CENT OF THE PIGMENT RECOVERED)
1:20000	0.0013	2
1:2000	0.013	37
1:200	0.13	93

In spite of these side reactions, which were rather emphasized above, iodine catalysis in general is a powerful and reliable tool in research dealing with the stereochemistry of polyenes. We shall give now some specific results.

(a) *Hydrocarbon carotenoids*: The composition of the equilibrium mixture of pigments formed reversibly by iodine catalysis from α -carotene, β -carotene, 5,6-dihydro- α -carotene, and 5,6-dihydro- β -carotene is summarized in table 9 (65, 66, 120).

For lycopene the following data are available (128):

Minutes.....	0	5	30	90	180
Ratio of all- <i>trans</i> to neo forms.....	100:0	82:18	71:29	57:43	53:47

A mixture of neolycopenes A and B behaved as follows:

Minutes.....	0	5	30	90
Ratio of all- <i>trans</i> to neo forms.....	0:100	13:87	19:81	30:70

(b) *Monohydroxy-carotenoids*: The behavior of some members of the cryptoxanthin set (113) is given in table 10. Gazanixanthin, a monohydroxy- γ -

TABLE 9

Relative colorimetric values of some members of hydrocarbon sets as formed by iodine catalysis in petroleum ether solution in daylight

Duration: 30 min.; for β -carotene, 60 min.

STEREoisomeric SET	STARTING MATERIAL	RELATIVE COLORIMETRIC VALUES (IN PER CENT OF PIGMENT RECOVERED)					
		Neo U	Neo V	Neo W	All-trans	Neo B	Neo forms C + D + E
α -Carotene...	Neo U	11	3.5	21	49.5	12	3
	Neo V	11.5	4	19.5	50	13	2
	Neo W	15.5	3.5	18.5	43.5	17	2
	All-trans	14.5	3	15.5	51.5	13	2.5
	Neo B	10.5	3	15	57	12.5	2
	Neo forms C + D + E	10	2.5	21	51.5	9.5	5.5
		Neo U	All-trans	Neo B	Neo E	Labile isomer	
β -Carotene.....	Neo U	24	47	24	3	2	
	All-trans	22	48	25	3	2	
	Neo B	21	51	23	3	2	
	Neo E	20	48	24	4	4	
	Labile isomer	18	45	16	13	8	
		Neo T	Neo U	All-trans	Neo A	Neo B	
5,6-Dihydro- α -carotene....	Neo T	23	15	54	8		
	Neo U	5	22	55	18		
	All-trans	4	20	56	16	4	
	Neo A	2	17	49	26	6	
	Neo B	1	19	64	11	5	
		Neo forms U + V	All-trans		Neo forms A + B + C		
5,6-Dihydro- β -carotene.....	Neo U	17	63		20		
	Neo V	17	69		14		
	All-trans	18	67		15		
	Neo A	21	64		15		
	Neo forms B + C	16	70		14		

TABLE 10

Relative photometric values of some members of the cryptoxanthin set as formed by iodine catalysis in petroleum ether solution in 60 min. at room temperature in daylight

STARTING MATERIAL	RELATIVE COLORIMETRIC VALUES (PER CENT OF PIGMENT RECOVERED)			
	Neo U	All-trans	Neo A	Neo B
Neo U.....	23	55	22	
All-trans.....	18	59	18	5
Neo A.....	20	57	23	
Neo B.....	21	55	17	7

carotene (71, 125), attained equilibrium within 15 min. under the conditions employed, and this time was also sufficient if iodine was added to the mixture of the isomers. The following figures refer to the catalysis of the all-*trans* form; the stereoisomers, the chromatographic separation of which is difficult, are given as Groups I and II.

Minutes of catalysis.....	5	15	30
Ratio of all- <i>trans</i> : Group I: Group II.....	72:20:8	56:29:15	56:29:15

(c) *Xanthophylls with two or more hydroxyl groups*: The behavior of lutein (128) and of zeaxanthin (106) is presented in tables 11 and 12. For taraxanthin

TABLE 11

Relative photometric values of some members of the lutein set formed by iodine catalysis in petroleum ether in 30 min. in daylight

STARTING MATERIAL	RELATIVE PHOTOMETRIC VALUES (PER CENT OF PIGMENT RECOVERED)		
	Neo A	Neo B	All- <i>trans</i>
Neo A.....	20	22	58
Neo B.....	20	24	56
All- <i>trans</i>	17	23	60

TABLE 12

Relative photometric values of some members of the zeaxanthin set formed by iodine catalysis in benzene in 30 min. in daylight

STARTING MATERIAL	RELATIVE PHOTOMETRIC VALUES (PER CENT OF PIGMENT RECOVERED)			
	Neo A	Neo B	Neo C	All- <i>trans</i>
Neo A.....	30	15	3	52
Neo B.....	10	19	3	68
Neo C.....	12	18	3	67
All- <i>trans</i>	10	21	3	66

only a few data are available (128). The esterification of the hydroxyls of xanthophylls leaves the iodine sensitivity essentially unaltered.

(d) *Ketones*: The behavior of all-*trans*-capsanthin in benzene solution is illustrated by the following experiment (105), which was carried out at 20°C.:

Minutes of catalysis.....	1	15	900
Ratio of all- <i>trans</i> : neo A: neo B: neo C...	78:8:11:3	69:15:11:5	66:23:7:4

The behavior of the neo forms is similar.

The following figures for capsanthin dipalmitate are typical; the same equilibrium was reached rapidly from both sides and remained constant over a long period of time.

STARTING MATERIAL	RATIO OF ALL- <i>trans</i> TO NEO FORMS			
	After 15 min.	After 30 min.	After 60 min.	After 24 hr.
All- <i>trans</i>	64:36	64:36	67:33	68:32
Neo forms A + B.....	64:36	66:34	66:34	66:34

Capsorubin in benzene isomerized in the following manner (105):

STARTING MATERIAL	RATIO OF ALL- <i>trans</i> TO NEO FORMS		
	After 15 min.	After 60 min.	After 15 hr.
All- <i>trans</i>	72:28	75:25	68:32
Neo forms.....	67:33	65:35	

Capsorubin dipalmitate in benzene after a 15-min. catalysis had isomerized to the extent of 26 per cent and did not isomerize further on standing for a day at 20°C.

4. *Cis-trans isomerization by acid catalysis*

The quality and quantity of the products which are formed by the action of acids on carotenoids depend upon the conditions, especially upon the strength and the concentration of the acid. In the extreme case total destruction and bleaching occurs, but under moderate conditions structural changes may take place which cannot be qualified as cleavage. To this category belong the reduction of carotenes with cold commercial concentrated hydriodic acid (66) and the formation of "desoxyluteins" in the boric acid melt, investigated by Sease and the author (126). Under still milder conditions which are, however, by no means equal for each carotenoid, spatial rearrangements chiefly take place.

The greater acid sensitivity of the xanthophylls is a well-known characteristic. Some xanthophylls or hydroxyketones (violaxanthin, fucoxanthin, capsanthin, capsorubin, etc.) give dark blue colorations with strong hydrochloric acid.

According to Kuhn, Winterstein, and Lederer (41) lutein is very sensitive toward small amounts of organic acids when solutions in methanol are refluxed; the rotatory power increases while the melting point decreases. These observations were confirmed and extended by Strain (82, 84). Simultaneously, an investigation by Quackenbush, Steenbock, and Peterson (69) showed the formation of several new polyene pigments from the xanthophylls of forage and from pure lutein under the influence of different acids. It is unknown, at present, which, if any, of these pigments were formed by steric changes. Some further observations have recently been made by Strain (84) on several xanthophylls, and by Strain and Manning (86) on fucoxanthin.

Acid isomerizations of carotenoids can be prevented by the addition of such organic bases as pyridine, quinoline, or dimethylaniline (Strain (84)).

In the author's laboratory, the acid catalysis of a polyene is usually carried out by shaking a petroleum ether or benzene solution with more or less con-

centrated aqueous acid under carbon dioxide, for example, for half an hour. The catalytic process is accelerated in this technique by the incessantly changing interface.

When β -carotene (66) was treated with commercial concentrated hydrochloric acid, it was converted into irreversible pigments to a slight extent only, and the composition of the stereoisomeric mixture recovered was as follows: all-*trans*: neo U:neo B:neo E:unnamed labile isomer = 50:23:23:3:1. The action of 28 per cent hydriodic acid upon α -carotene gave this mixture: all-*trans*: neo U:neo V:neo W:neo B:neo C = 49:13:2:15:8:9:4. The members of both sets were destroyed by the action of 56 per cent hydriodic acid. The acid isomerization behavior of 5,6-dihydro- α - and β -carotenes prepared by Polgár and the author (66) was similar. Pro- γ -carotene (122), after interaction with concentrated hydrochloric acid for 30 min., yielded a stereoisomeric mixture of the γ -carotene set in which unchanged starting material was absent; some irreversible products were formed.

5. Photochemical *cis-trans* isomerization

All carotenoid solutions which have been investigated up to the present time are photosensitive, but the degree to which they possess this characteristic shows considerable variation. It is dependent both on the chemical and on the spatial structure. Visible light is much more effective than wave lengths in the ultra-violet region. Most of the experiments have been conducted by exposing the pigment solutions to intense sunshine ("insolation") in transparent quartz tubes from which the air had been displaced with carbon dioxide.

In order to differentiate the photo-isomerization from the thermal process, the temperature should be noted and, if necessary, a cooling device should be employed.

Of course, *cis-trans* isomerization is only one of the processes which take place. It competes with the more or less rapid destruction of pigment which is probably promoted by traces of oxygen and by accidental catalysts. We have observed complete bleaching in some cases. Thus a sharp differentiation between cleavage and *cis-trans* rearrangement is necessary and may be achieved by chromatographing the irradiated solution and subsequently testing the reversibility of the pigment in each zone. The partition behavior should also be checked.

If an all-*trans* carotenoid is exposed to light, both *trans* $\rightarrow$ *cis* isomerization and destruction will cause a decrease in the color intensity. When a pigment which contains only one or two *cis* double bonds is irradiated, the experimental conditions will determine whether the color-decreasing effect of destruction or the color-increasing effect of *cis* $\rightarrow$ *trans* isomerization will prevail. However, if a poly-*cis* compound is exposed to sunlight for a short time, the tremendous increase in the colorimetric value caused by steric changes will predominate over any other effect. Thus, it was observed in collaboration with LeRosen, Schroeder, Polgár, and Pauling (116) that very dilute solutions of prolycopene or pro- γ -carotene, which were almost colorless, turned intensely yellow after exposure to sunshine for a few minutes. This change, which in the visible region

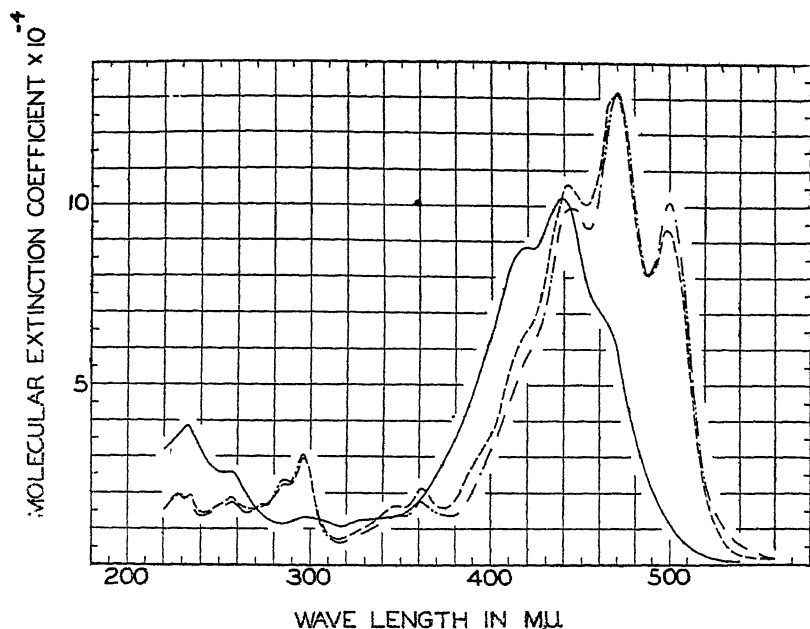


FIG. 4. Photo-isomerization of polycopene in hexane: —, fresh solution of polycopene; ---, mixture of stereoisomers after insolation for 20 min.; — · —, mixture of stereoisomers after insolation for 60 min. (From J. Am. Chem. Soc. **65**, 1945 (1943))

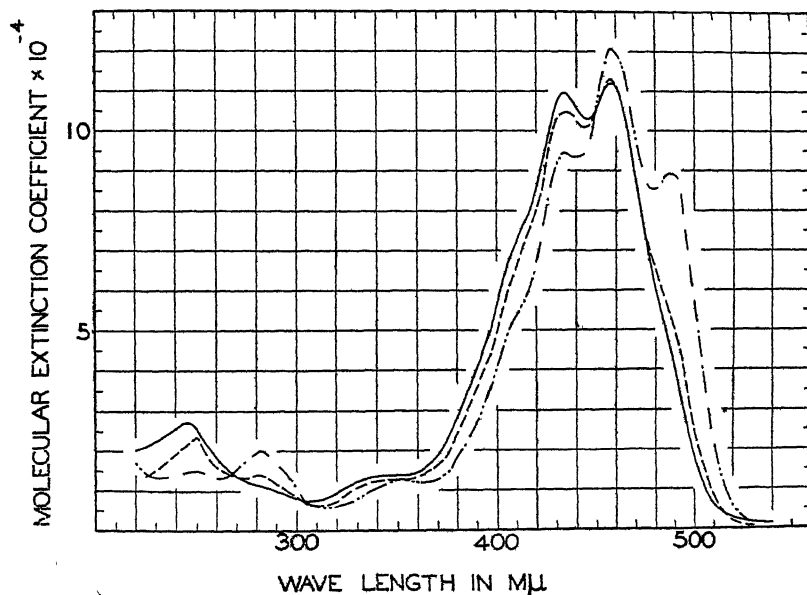


FIG. 5. Photo-isomerization of pro- γ -carotene in hexane: —, fresh solution of pro- γ -carotene; ---, mixture of stereoisomers after insolation for 5 min.; — · —, mixture of stereoisomers after insolation for 30 min. (From J. Am. Chem. Soc. **65**, 1944 (1943))

includes both a great increase in the extinction values and a substantial shift toward longer wave lengths, is illustrated in figures 4 and 5 (p. 291).

The photochemical behavior of all-*trans*- α - and - β -carotenes and of their main known isomers is demonstrated in tables 13 and 14 (Polgár and the author (120)). These tables show that the all-*trans* configuration in carotenes is by far the least photosensitive. Some of the neo forms decreased rapidly in quantity upon exposure to sunshine; however, the pigment mixture formed from a comparatively photostable isomer, neo- α -carotene U, still contained 92 per cent of this isomer after irradiation for 45 min. with a 1000-watt Mazda bulb from a distance of 10

TABLE 13

Relative colorimetric values of all-trans- α -carotene and some of its stereoisomers formed by 45 min. insolation (in petroleum ether)

STARTING MATERIAL	RELATIVE COLORIMETRIC VALUES (PER CENT OF THE PIGMENT RECOVERED)								
	Neo U	Neo V	Neo W	Neo X	Neo Y	All- <i>trans</i>	Neo A	Neo B	Neo C + D etc.
Neo U.....	64.5	1.5	3.5	2	3	24		1.5	
Neo V.....	33	43	4	2		16		2	
Neo W.....	7.5	5	32.5	1		41	4	7.5	1.5
All- <i>trans</i>	1.5		2			93.5		2.5	0.5
Neo B.....	1.5		34			56.5		8	

TABLE 14

Relative colorimetric values of all-trans- β -carotene and of some of its stereoisomers formed by 45 min. insolation (in petroleum ether)

STARTING MATERIAL	RELATIVE COLORIMETRIC VALUES (PER CENT OF PIGMENT RECOVERED)				
	Neo U	Neo V	All- <i>trans</i>	Neo B	Neo C + D etc.
Neo U	36.5		55	6	2.5
All- <i>trans</i>	1		98	1	
Neo B... ..	27	2.5	60	5	5.5

cm. After exposure to an ultraviolet lamp (Hanovia, scientific type) for 30 min. the corresponding value was 98 per cent.

That it is not yet possible to correlate structural features with the degree of photosensitivity is demonstrated by the behavior of cryptoxanthin and zeaxanthin. As shown below in table 15, all-*trans*-zeaxanthin, $\text{HOC}_{40}\text{H}_{54}\text{OH}$, in spite of its symmetrical structure, is much more easily bleached by sunshine than is all-*trans*-cryptoxanthin, $\text{C}_{40}\text{H}_{55}\text{OH}$ (Lemmon and the author (113)).

It is advisable to determine by such preliminary experiments the optimum duration of the exposure for the purpose of a study of the photo-isomerization. For cryptoxanthin insolation for 45 min. was found to be adequate (unchanged all-*trans*: neo A = 86:14), and for zeaxanthin insolation for 15 min. Insolation of the latter pigment for 5 min. produced only a slight stereoisomerization; on

the other hand, after exposure for 30 min. and subsequent adsorption analysis, nearly the whole recovered pigment passed into the chromatographic filtrate, under conditions under which any member of the zeaxanthin set would have remained adsorbed in the column. After 15 min. insolation of zeaxanthin (in benzene) the recovered pigment was composed of 48 per cent unchanged all-*trans* form, 11 per cent neozeaxanthins, 11 per cent minor pigments, and 30 per cent of an intensely colored, crystallizable polyene which in the visual spectroscopy was similar to zeaxanthin, but on the basis of its partly epiphasic behavior did not belong to that set.

D. SOME PROPERTIES OF CAROTENOIDS CONTAINING *cis* DOUBLE BONDS

The physical character of carotenoids with *cis* double bonds is essentially analogous to that of simpler ethylenic structures.

If an all-*trans* pigment undergoes a steric rearrangement, the color intensity of the solution is decreased and the solubility of the isomers formed is increased. The melting points of crystallizable isomers are lower than that of the all-*trans*

TABLE 15

Loss of color intensity of benzene solutions of cryptoxanthin and zeaxanthin on exposure to sunshine

DURATION OF INSOLATION <i>minutes</i>	LOSS OF COLOR INTENSITY (IN PER CENT OF THE INITIAL VALUE)	
	Cryptoxanthin	Zeaxanthin
15	6	21
30	17	39
45	26	63
60	36	88

form. The poly-*cis* compounds, polycopene and pro- γ -carotene, and some other isomers which contain fewer *cis* double bonds, crystallize readily, but this tendency is markedly diminished, for example, in some members of hydrocarbon sets. Those stereoisomers of α -carotene and β -carotene which are adsorbed below the all-*trans* compound are chromatographically heterogeneous in the sense that fresh solutions of the crystals formed several zones on the column. Such is the situation also with "pseudo- α -carotene" (10), "neocarotene" (11), and "neo- α -carotene" (11). Neolycopene A gave all-*trans* crystals under the conditions employed (129).

Noteworthy is the considerable thermal stability of some stereoisomers when refluxed in solution at 60–80°C. This is evident from various data given below, especially those which are concerned with polycopene and pro- γ -carotene.

When asymmetric carbon atoms are present, the rotatory power is obviously dependent on *cis-trans* isomerism. The values may undergo alterations in both directions if the all-*trans* molecule is bent. For example, the initial specific levorotation of all-*trans*-zeaxanthin, -42.5° , changed into a dextrorotation upon

either refluxing or iodine catalysis. This inversion proved the presence of a strongly dextrorotatory stereoisomer in the mixture (106). The specific rotation of all-*trans*-gazaniaxanthin, $\pm 0^\circ$, increased to $+150^\circ$ when isomerization of the compound was catalyzed with iodine (125). Some data were also reported by Strain (82).

In several of the sets listed in table 16 one stereoisomer surpasses all the other members of the set tested so far in rotatory power: for example, neozeaxanthin A,

TABLE 16
Dependence of the specific rotatory power on the configuration of some carotenoids

STEREISOOMIC SET	SOLVENT	MEMBER OF THE SET	$[\alpha]_{\text{Cd}}^{25}$ or $[\alpha]_{\text{C}}^{20}$	REFERENCES
α -Carotene	Chloroform	All- <i>trans</i> Neo U	$+359^\circ$ $+224^\circ$	(120)
Gazaniaxanthin...	Petroleum ether	All- <i>trans</i> Neo-Group I	$\pm 0^\circ$ $+220^\circ$	(125)
Zeaxanthin	Chloroform	All- <i>trans</i> Neo A Neo B	-42.5° $+120^\circ$ $\pm 0^\circ$	(106)
Capsanthin	Benzene	All- <i>trans</i> Neo A Neo B Neo C	$\pm 0^\circ$ $+89^\circ$ $+21^\circ$ $+27^\circ$	(105)
Capsanthin dipalmitate.....	Petroleum ether	All- <i>trans</i> Neo A Neo B	-30° -22° -20°	(105)
Capsorubin	Benzene	All- <i>trans</i> Neo A Neo B	$\pm 0^\circ$ -134° -69°	(105)
Capsorubin dipalmitate.....	Petroleum ether	All- <i>trans</i> Neo A Neo B	$\pm 0^\circ$ -75° -15°	(105)

neocapsanthin A, neocapsorubin A, neocapsorubin dipalmitate A, and the neogazaniaxanthins belonging to Group I. Each of these pigments differs from the all-*trans* form by only a few millimicrons in its visually determined spectral maxima; and furthermore, they adsorb immediately below the all-*trans* compound in the Tswett column.

It remains to be seen whether or not a bending of an all-*trans* carotenoid molecule at or near the center of the chromophore necessarily involves a substantial increase of molecular asymmetry as expressed by increased rotations.

E. *Cis-trans* ISOMERIZATION AND SPECTRAL CHARACTERISTICS1. Alterations in the visible region by bending of the all-*trans* molecule

The most spectacular changes caused by *trans* $\rightarrow$ *cis* rotations of an all-*trans* carotenoid are the alterations of adsorption affinity and light extinction. While, however, the adsorption forces may either increase or decrease as a consequence of *trans* $\rightarrow$ *cis* rotations, the shift of the spectral maxima in the visible region

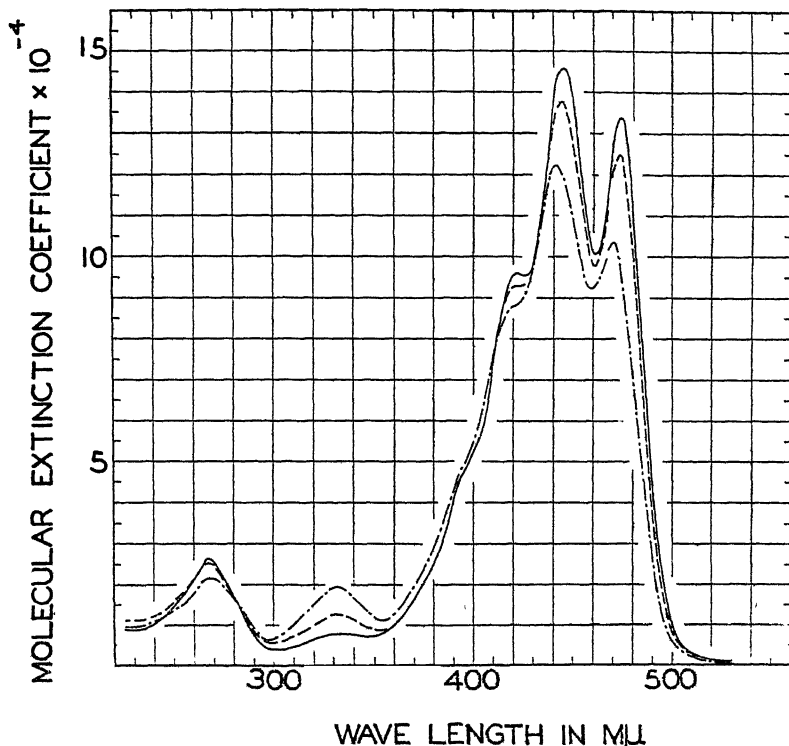


FIG. 6. Molecular extinction curves of α -carotene in hexane: —, fresh solution of the all-*trans* compound; ---, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **65**, 1522 (1943))

follows a uniform direction in every case. If the solution is concentrated enough, this becomes manifest even to the naked eye by a spectacular decrease in color intensity. The bands observed in the visual spectroscop are then shifted toward shorter wave lengths relative to the corresponding bands of the all-*trans* form. Such a change can be demonstrated by adding a drop of dilute iodine solution to the pigment in a spectroscopic cell while an observer is watching.

Spectroscopic changes in the opposite direction occur if the isomerization of a pigment which has *cis* bonds is catalyzed by iodine. Depending on the con-

figuration, this shift may be larger or smaller than that observed with the all-*trans* form, but in no case will the spectrum of the all-*trans* isomer be reached, since an equilibrium mixture is formed.

Stereoisomerization also causes the molecular extinction curves in the visible region to undergo a characteristic change. If an all-*trans* carotenoid is refluxed or catalyzed, the extinctions decrease. Furthermore, a shift toward shorter wave lengths is observed. The extinction curve of a fresh solution of a carotenoid which has *cis* double bonds, however, is shifted both in position and in intensities in the opposite direction when treated with iodine.

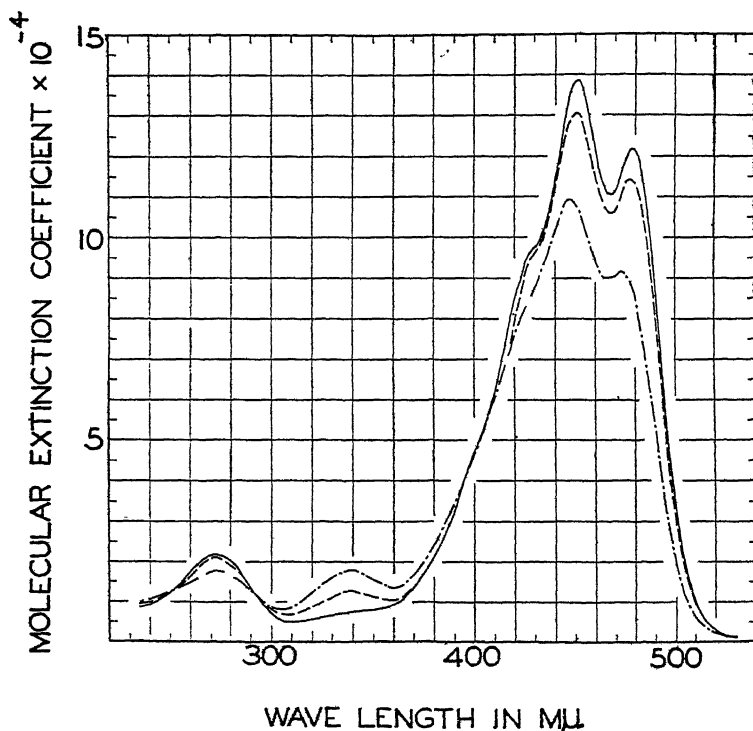


FIG. 7. Molecular extinction curves of β -carotene in hexane: —, fresh solution of the all-*trans* compound; — — —, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 65, 1523 (1943))

All these observations are in accordance with theoretical treatments presented by Pauling (62, 63), Mulliken (56, 57, 58), and Lewis and Calvin (46).

In a conjugated double-bond system, the single bonds possess sufficient double bond character to maintain a coplanar system, but these tendencies may be overcome by steric changes. The hydrogen atoms of CH groups adjacent to a double bond overlap somewhat in the *cis* configuration (figure 1) and the consequent interatomic repulsion tends to push the *cis*-isomer out of coplanarity.

This interference with the conjugation shifts the spectral bands to shorter wave lengths (Pauling (62)).

We see that the postulate is well founded both from the experimental and from the theoretical side and that among all members of a stereoisomeric set the all-*trans* compound must be expected to show the greatest light extinction in the visible region.

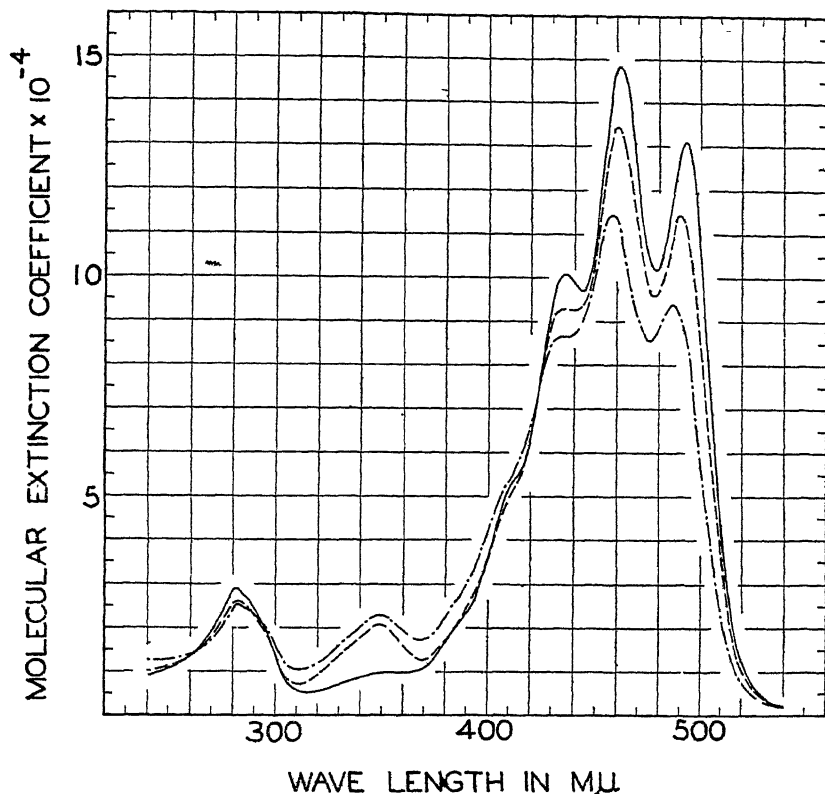


FIG. 8. Molecular extinction curves of γ -carotene in hexane; —, fresh solution of the all-*trans* compound; — — —, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **65**, 1941 (1943))

Visual spectroscopic data which illustrate the dependence of the position of maxima on the configuration can be found in numerous papers, the first of which was published by Gillam and El Ridi (10). More detailed information for the spectral region down to 380 $m\mu$ is provided by the extinction curves of natural and isomerized carotenoids which have been published by Strain (81, 82, 83), Beadle and Zscheile (1), White, Zscheile, and Brunson (97), and by White, Brunson, and Zscheile (96); cf. (5, 8, 21a, 21b, 23a, 36, 52a, 54, 55, 73, 76, 130, 131, 132).

2. Alterations in the ultraviolet region by bending of the all-trans molecule: the *cis*-peak effect

The study of the alterations in spectral curves of carotenoids which attend *trans* $\rightarrow$ *cis* rotations have been extended farther into the ultraviolet region only recently in collaboration with Polgár (119).

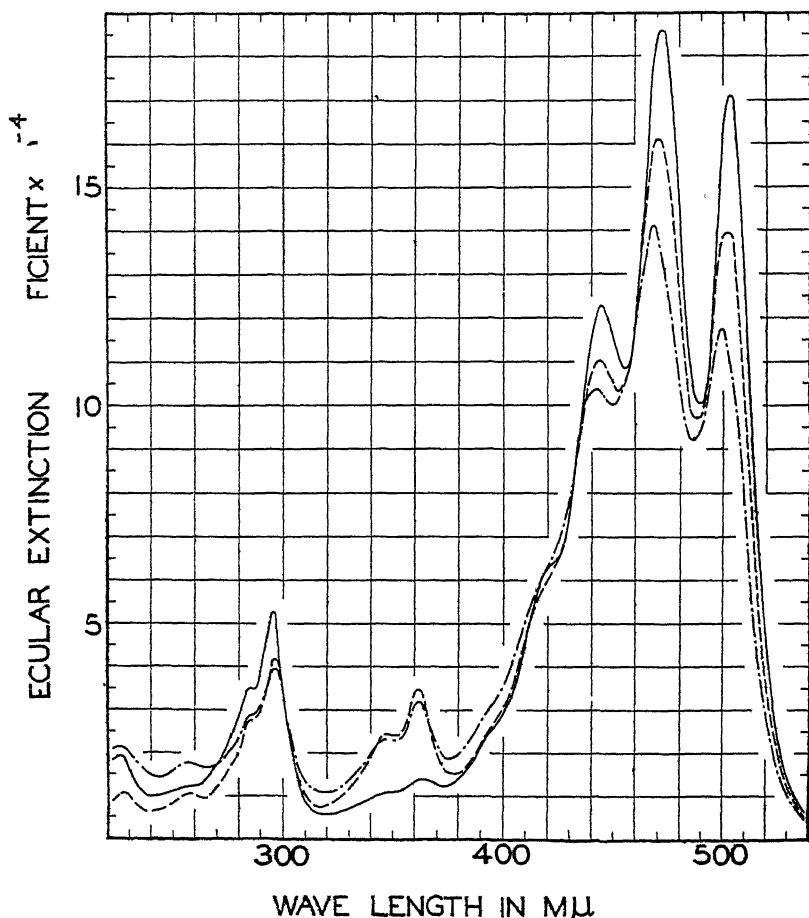


FIG. 9. Molecular extinction curves of lycopene in hexane: —, fresh solution of the all-trans compound; — — —, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 65, 1942 (1943))

It has been customary in our laboratory to characterize each carotenoid studied from the stereochemical viewpoint by three molecular extinction curves which extend if possible to 220 $m\mu$; first, a fresh solution is examined, then the same solution after refluxing for 45–60 min., and finally another portion after standing $\frac{1}{2}$ to 1 hr. in scattered light (fluorescent lamp) in the presence of iodine (1 to 2 per cent of the pigment). Although the thermal or catalytic treatment

decreases the extinction of an all-*trans* carotenoid in the visual region as well as in the far ultraviolet (260 to 320 $m\mu$ in hexane), a new marked maximum develops at a somewhat longer wave-length range in the ultraviolet, that is, somewhere between 320 and 380 $m\mu$ where all-*trans* carotenoids show a very slight elevation at most. The new maximum has been termed the "*cis*-peak" and the range of wave lengths in which it appears, the "*cis*-peak region". The differ-

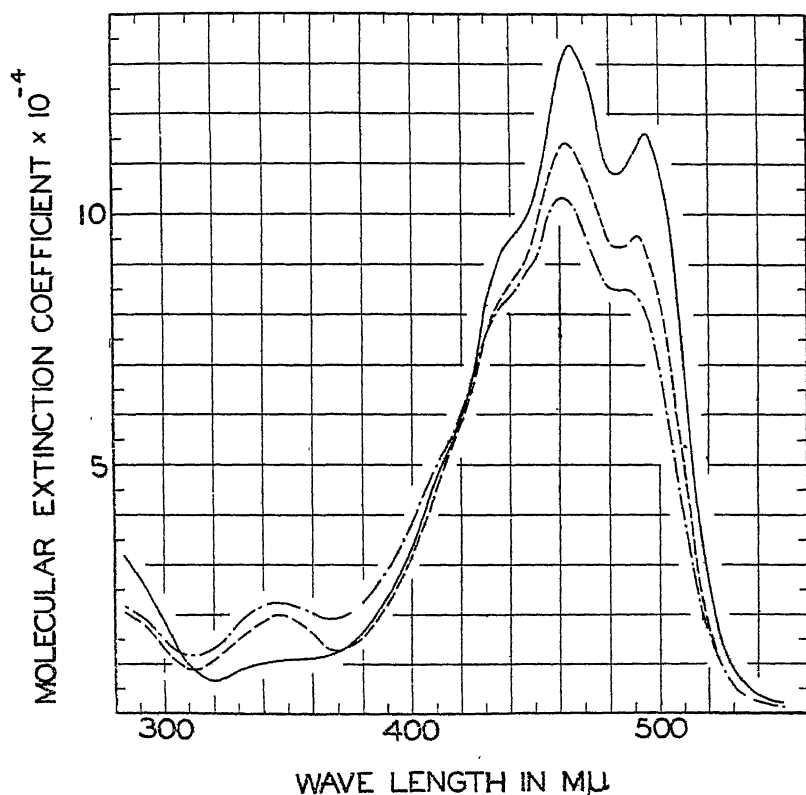


Fig. 10. Molecular extinction curves of cryptoxanthin in benzene: —, fresh solution of the all-*trans* compound; ---, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 318 (1944))

ence between the total extinction of an isomerized pigment and that of its all-*trans* form at the wave length of the peak is the "*cis*-peak effect" (figures 6 to 13).

The *cis*-peak has a definite position in the extinction curve (table 17). The difference between its wave length and that of the longest wave-length maximum of the all-*trans* form is practically a constant, that is, 142 $m\mu$ ($\pm 2 m\mu$) in hexane. It is understandable that the relative position of these two maxima is not markedly altered by the presence of functional groups which are located on the periphery of the molecule.

Maxima observed earlier by McNicholas (50) and by Miller (52, 52a) at wave lengths which correspond to the *cis*-peak were ascribed to oxidation. This was the only reasonable explanation at a time when the stereochemistry of carotenoids was unexplored.

In other cases *cis*-peaks have been represented as a definite feature of the all-*trans* curve, but it is evident that a partial stereoisomerization in the solu-

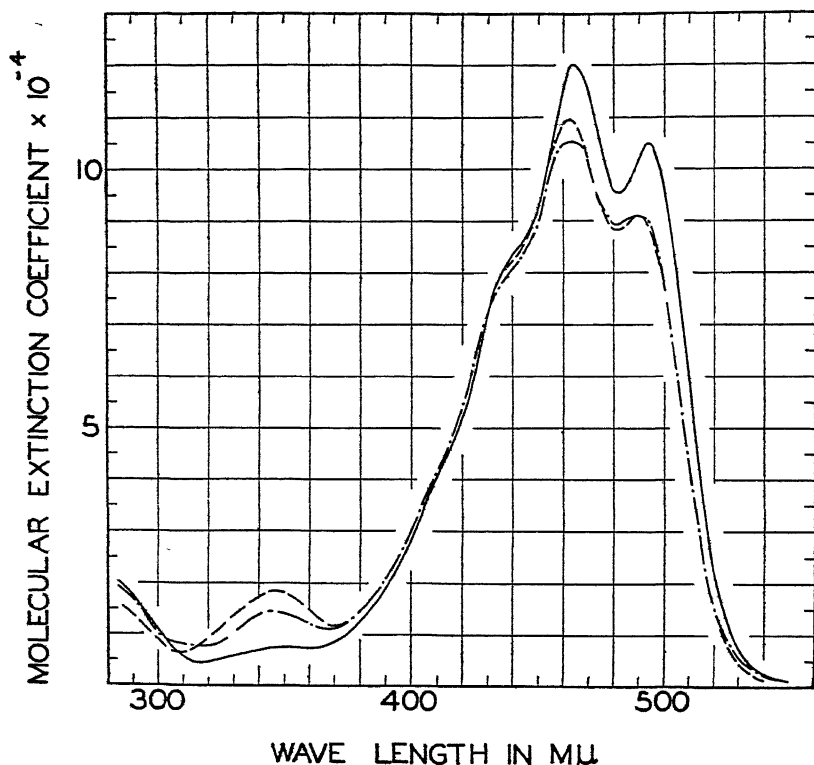


FIG. 11. Molecular extinction curves of zeaxanthin in benzene: —, fresh solution of the all-*trans* compound; — — —, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **66**, 319 (1944))

tions used was responsible for the effect (*cf.*, for example, some of the figures in reference 23a).

In complete darkness most carotenoids do not develop *cis*-peaks in the presence of iodine within an hour or so. However, a light impulse as short as 5 sec. may be sufficient to produce an effect which is a third or a half of the total peak. Examples are given in figures 14–15 and in table 18 (p. 305). Long illumination in intense light may lead eventually to destructive processes.

The extinction values discussed in the present review refer mainly to benzene,

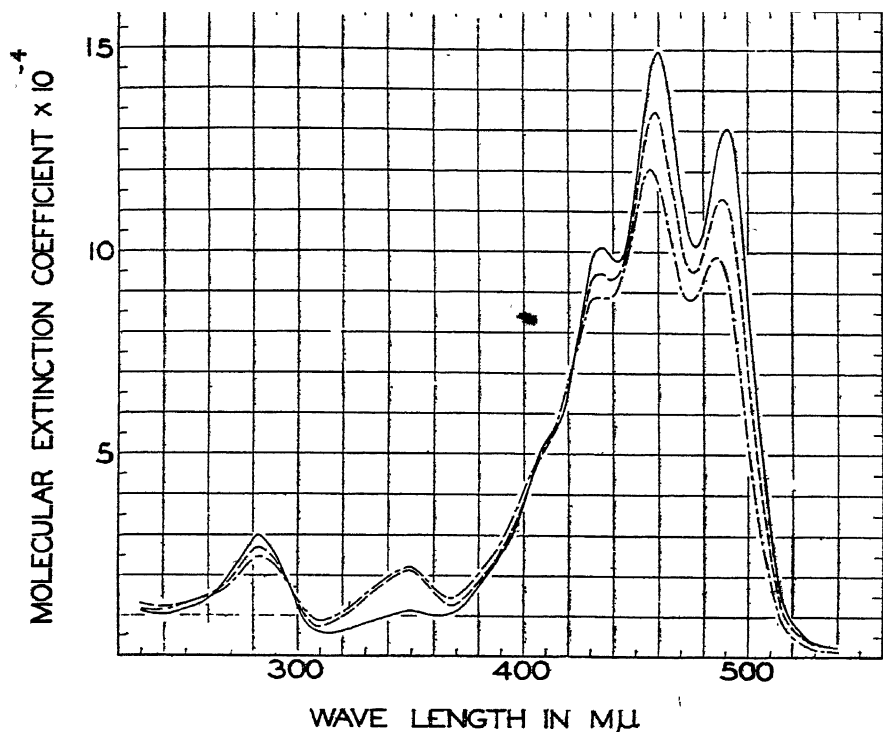


FIG. 12. Molecular extinction curves of gazaniaxanthin in hexane: —, fresh solution of the all-*trans* compound; ---, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 65, 1536 (1943))

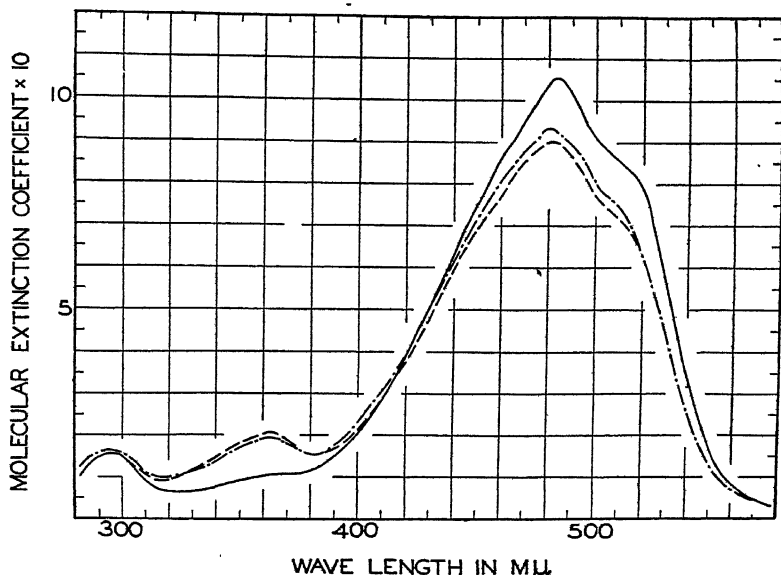


FIG. 13. Molecular extinction curves of capsanthin in benzene: —, fresh solution of the all-*trans* compound; ---, mixture of stereoisomers after refluxing in darkness for 45 min. — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 187 (1944))

TABLE 17

Position of the *cis*-peak in the spectral curve of stereoisomeric equilibrium mixtures of some C_{40} -carotenoids obtained after iodine catalysis (119)

PIGMENT	POSITION OF THE <i>cis</i> -PEAK	DISTANCE BETWEEN <i>cis</i> -PEAK AND LONGEST WAVE-LENGTH MAXIMUM OF THE ALL- <i>trans</i> FORM
In hexane solution		
5,6-Dihydro- α -carotene (66).....	$m\mu$ 329	$m\mu$ 140
5,6-Dihydro- β -carotene (66).....	331	143.5
α -Carotene.....	331	143
Lutein.....	331	143
β -Carotene.....	339.5	139.5
Cryptoxanthin.....	339	141
Physalien.....	338	141
γ -Carotene.....	349.5	143.5
Gazaniaxanthin (71, 125).....	349	142
Lycopene.....	362	141.5
Capsanthin.....	355	143
Celaxanthin (44).....	380	144
In benzene solution		
Cryptoxanthin.....	348	146
Zeaxanthin.....	348	145
Capsanthin.....	363	145

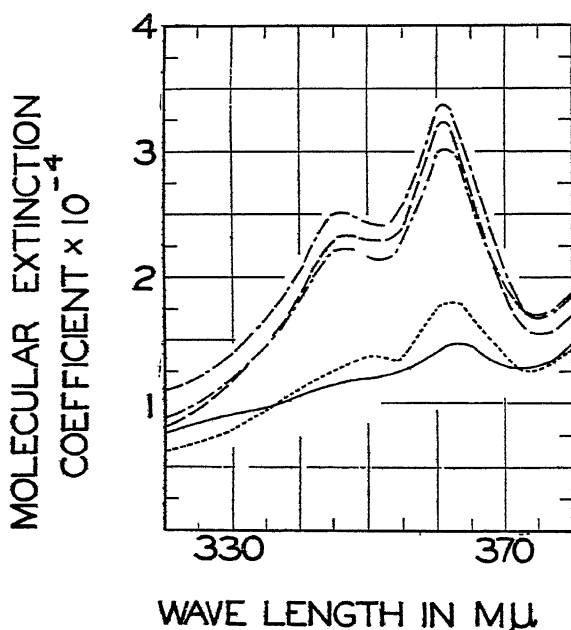


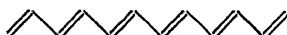
FIG. 14. Influence of illumination on the development of the *cis*-peak in an iodine-catalyzed solution of all-*trans*-lycopene in hexane: ····, molecular extinction curve after zero seconds; —, after 5 sec.; — — —, after 30 sec.; — · —, after 15 min. illumination. The full line denotes the curve taken before the addition of iodine, without illumination. (From J. Am. Chem. Soc. 66, 189 (1944))

petroleum ether, or hexane solutions. Lycopene (and some other pigments) when refluxed or catalyzed with iodine in carbon disulfide show elevated but remarkably flat extinction curves in the *cis*-peak region (unpublished work by Polgár and the author).

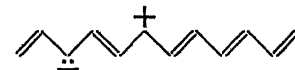
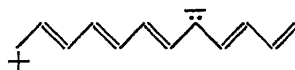
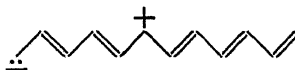
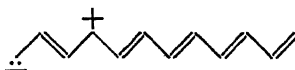
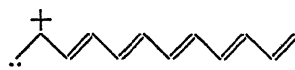
3. Interpretation of the *cis*-peak effect

The following theoretical considerations have recently been given by Pauling *et al.* (116) and are partly based on earlier treatments (46, 47, 56, 57, 58, 62, 63, 64).

The highly unsaturated chromophore in carotenoids may be discussed in terms of a system resonating among the conventional structure



and a great number of ionic structures, such as:



The conventional structure makes the most important contribution to the normal state, whereas the ionic structures contribute in the main to excited states.

As we have seen, in the extinction curves of carotenoids three main regions must be differentiated, also from the practical viewpoint of the spectroscopist, *viz.*: (1) the region of the extraordinarily strong extinction in the visible region, which constitutes the fundamental absorption band, having a more or less pronounced vibrational fine structure; (2) the "*cis*-peak region" in the near ultraviolet (first overtone); and (3) a region in the far ultraviolet (second overtone). It is assumed that these absorption bands result from transitions from the normal electronic state to the three excited states, to wit, $0 \rightarrow 1$, $0 \rightarrow 2$, and $0 \rightarrow 3$, respectively. Following Lewis and Calvin (46) we may compare these with the classical modes of vibration of mobile "unsaturation" electrons along the chain.

The observed spectral bands can be correlated with the following manners of oscillation:

Fundamental band: The electrons tend to concentrate first near one end and then near the other of the conjugated system. This simple oscillation would, according to classical electromagnetic theory, result from the absorption of light of the proper frequency because of interaction of the electric vector of the light and the regularly reversing dipole moment.

Band in the "cis-peak region": This results from the oscillation of the electrons from the two ends of the conjugated system toward the middle and from the middle toward the two ends.

Band in the far ultraviolet: This involves concentration of the electrons alternately in the first and third and the second and fourth quarters of the system.

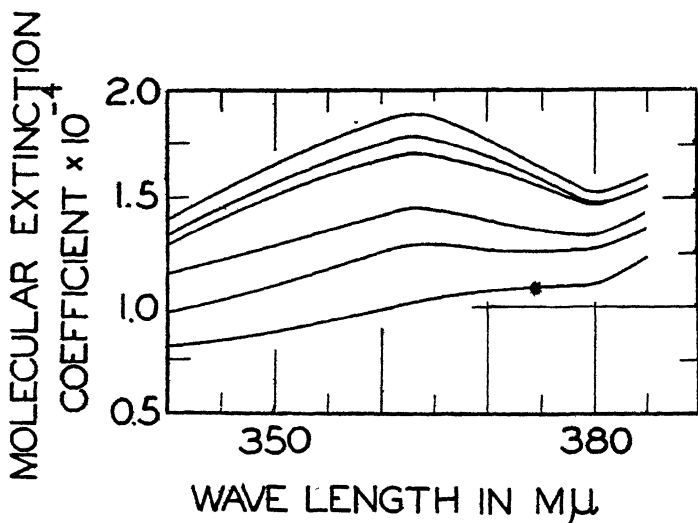


FIG. 15. Influence of illumination on the development of the *cis*-peak in an iodine-catalyzed solution of all-*trans*-capsanthin in benzene. Molecular extinction curves from bottom to top: after 0 sec., 5 sec., 30 sec., 2.5 min., 15 min., and 30 min. illumination. (From J. Am. Chem. Soc. **66**, 188 (1944))

The intensity of this maximum corresponding to the transition $0 \rightarrow 3$ may be expected to vary in rough proportionality to that of the fundamental band, and this is confirmed by experiments.

The intensity of a spectral band is proportional to the square of the corresponding dipole moment, and hence essentially to the square of the length of the conjugated system (57, 58, 62). Consequently, the maximum intensity of the fundamental band would be shown by the all-*trans* member of a set. All stereoisomers which have a vertical plane of symmetry have a distance between the two ends of the conjugated system smaller than the all-*trans* isomer by the factor $\cos \alpha$, with $\alpha = 27^\circ 22'$ (if the carbon bond angle along the chain is $125^\circ 16'$). The intensities for all stereoisomers permitted by the given postulates should lie between these extremes.

TABLE 18

Influence of light on the development of the cis-peak in iodine-catalyzed solutions of all-trans carotenoids (67, 118, 120)

(Mazda fluorescent lamp at 60 cm. distance)

PIGMENT	DURATION OF THE ILLUMINATION	MOLECULAR EXTINCTION COEFFICIENT AT THE LONGEST WAVE-LENGTH MAXIMUM $E_{1\text{cm.}} \times 10^{-4}$	MOLECULAR EXTINCTION COEFFICIENT AT THE <i>cis</i> -PEAK WAVE LENGTH $E_{1\text{cm.}} \times 10^{-4}$	DIFFERENCE OF THE MOLECULAR EXTINCTION COEFFICIENT FROM THE 0 SEC. VALUE, EXPRESSED IN PER CENT OF THE GREATEST CHANGE OBSERVED IN EACH SET
α -Carotene (in hexane)...	0 sec.	14.6	0.8(6)	0
	30 sec.	13.7	1.94	75
	2½ min.	13.4	2.11	87
	15 min.	13.2	2.16	90
	30 min.	12.7	2.30	100
	60 min.	11.3	2.26	97
β -Carotene (in hexane) ...	0 sec.	14.2	0.8(7)	0
	30 sec.	13.2	1.95	80
	2½ min.	13.0	2.04	87
	15 min.	12.3	2.22	100
	30 min.	10.9	2.22	100
Lycopene (in hexane)....	0 sec.	18.6	1.47	0
	5 sec.	15.5	3.2	92
	30 sec.	15.6	3.4	100
	2½ min.	15.4	3.2	92
	15 min.	14.0	3.0	81
Cryptoxanthin (in benzene).....	0 sec.	13.4	0.70	0
	5 sec.	13.1	0.81	10
	30 sec.	12.7	1.16	42
	2½ min.	12.0	1.62	84
	15 min.	11.0	1.77	98
	30 min.	10.0	1.79	100
Zeaxanthin (in benzene)..	0 sec.	12.0	0.89	0
	5 sec.	11.8	1.25	42
	30 sec.	11.4	1.47	68
	2½ min.	11.1	1.64	88
	15 min.	10.8	1.74	100
	30 min.	10.5	1.71	97
	60 min.	10.1	1.74	100
Capsanthin (in benzene)..	0 sec.	9.9	1.02	0
	5 sec.	9.8	1.29	31
	30 sec.	9.5	1.47	51
	2½ min.	9.3	1.72	80
	15 min.	9.0	1.80	89
	30 min.	9.0	1.89	99
	60 min.	8.9	1.90	100

The nature of the $0 \rightarrow 2$ oscillation, which was discussed by Mulliken (57, 58), is such that it gives rise to no dipole moment and hence to no absorption band for the all-*trans* molecule or any other molecule with a center of symmetry. However, that isomer which has one centrally located *cis* double bond possesses a dipole moment for the transition $0 \rightarrow 2$ which is perpendicular to the straight line between the ends of the conjugated system instead of parallel to it. This isomer would show the highest *cis*-peak. As a rough approximation the intensity of the *cis*-peak of any member of a stereoisomeric set can be taken proportional to the square of the distance between the center of the conjugated system and the mid-point of the straight line between its ends. Accordingly, only a few of the stereoisomers possible can have *cis*-peaks approaching in height that of the isomer with one *cis* bond in the center.

If these theoretical viewpoints are correct and if we start at the center of an all-*trans* chromophore and perform successive *trans* $\rightarrow$ *cis* rotations, then the *cis*-peak must first rise and then fall again as the molecule bends and straightens out. A continuous rise of the peak during these rearrangements is not to be expected.

Starting materials for an experimental test of these two alternatives cannot yet be prepared artificially, since poly-*cis* compounds have not been observed when a pigment is isomerized by current methods. However, nature has provided two such compounds, prolycopene (45) and pro- γ -carotene (122), which have already been discussed. In the visible region, petroleum ether solutions of these pigments show maxima at $35\text{ m}\mu$ and $31\text{ m}\mu$ shorter wave lengths than those of the maxima of the corresponding all-*trans* pigments, lycopene and γ -carotene.

It was first assumed (117) that prolycopene is an all-*cis* isomer, but melting of its crystals produced a new pigment with still shorter wave-length maxima in the visual region; therefore, the all-*cis* configuration is claimed for the latter pigment. It is very probable that six of the seven stereochemically available double bonds are *cis* in prolycopene and five of the six such bonds in pro- γ -carotene. The extent of the visually observed spectral shift in the last possible *trans* $\rightarrow$ *cis* rotation is approximately the same as observed in the first step. In petroleum ether solutions the following longest wave-length maxima were measured ($\pm 1\text{ m}\mu$):

All- <i>trans</i> -lycopene.....	504.5 $\text{m}\mu$	} $\Delta = 4.5\text{ m}\mu$
Neolycopene A.....	500	
Prolycopene.....	470.5	} $\Delta = 4.5\text{ m}\mu$
All- <i>cis</i> -lycopene.....	466	

Experiments carried out in collaboration with LeRosen, Schroeder, Polgár, and Pauling (116) showed that the extinction curves of the pro-carotenoids and of all-*cis*-lycopene are nearly as flat in the *cis*-peak region as the curve of the all-*trans* form (figures 16 to 18). These curves confirm that the theoretical viewpoints suggested are essentially correct and that a bending of the all-*trans* molecule, especially in the middle of the conjugated system, is mainly responsible for the appearance of a high *cis*-peak.

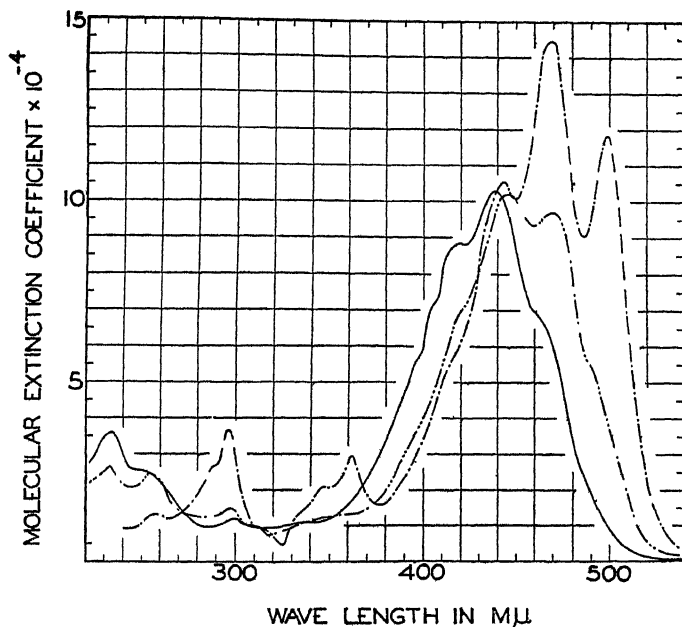


Fig. 16. Molecular extinction curves of polycyclopene and an unnamed crystalline stereoisomer in hexane: —, fresh solution of polycyclopene; ---, fresh solution of the isomer; — · —, mixture of stereoisomers in the polycyclopene solution after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **65**, 1943 (1943))

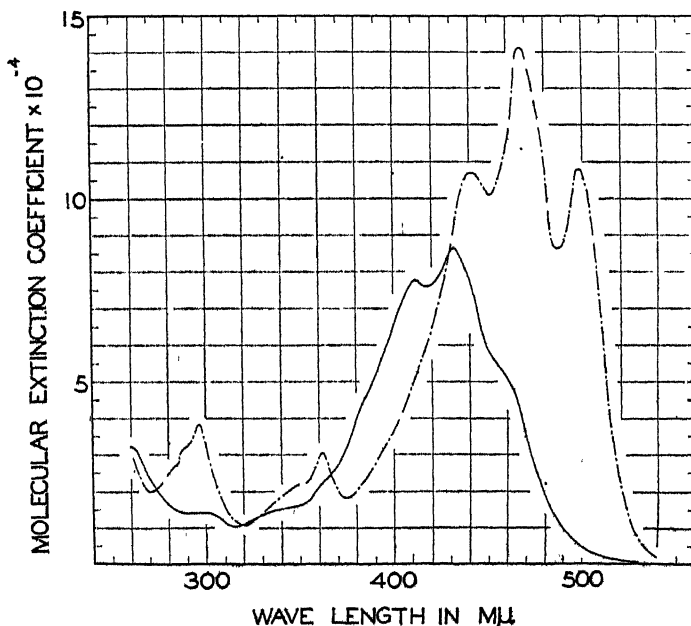


Fig. 17. Molecular extinction curves of all-*cis*-lycopenene in hexane: —, fresh solution of all-*cis*-lycopenene; ---, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **65**, 1943 (1943))

The spectral phenomena which can be used for a rapid diagnosis of a given stereochemical situation are summarized below in table 19.

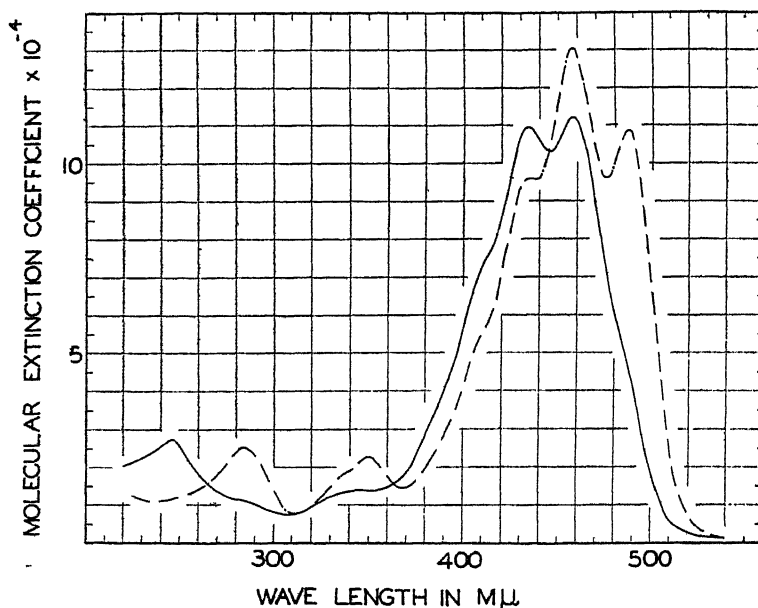


FIG. 18. Molecular extinction curves of pro- γ -carotene in hexane: —, fresh solution of pro- γ -carotene; ---, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **65**, 1941 (1943))

TABLE 19

Types of spectroscopic phenomena in the cis-peak and visible regions observed upon iodine catalysis

CONFIGURATION	SHAPE OF THE EXTINCTION CURVE IN THE <i>cis</i> -PEAK REGION	CHANGE OF EXTINCTION UPON IODINE CATALYSIS	
		In the visible region	In the <i>cis</i> -peak region
All- <i>trans</i>	Flat	Decrease	Increase
Central mono- <i>cis</i>	High peak	Increase	Decrease
All- <i>cis</i> or all-but-one- <i>cis</i>	Flat	Great increase	Increase

F. ASSIGNMENT OF CONFIGURATIONS

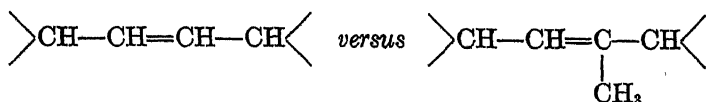
We shall now investigate to what extent it is possible at present to assign definite spatial structures to stereoisomers of carotenoids. This task is two-fold: in each case the number of the *cis* double bonds must be found and their position in the chromophore must be determined. Two different approaches to such work will be now discussed: first, considerations based on thermal (or photochemical) stereoisomerization which have so far been employed to only a

small extent, and second, considerations based on spectral phenomena, which constitute the main basis for the assignment of configurations.

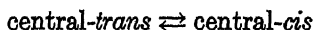
1. Thermal and photochemical isomerization methods

Some postulates concerning these methods have recently been discussed (116, 120).

According to the theory of conjugated systems, each of the double bonds of the system loses some of its double-bond character to the adjoining single bonds, the amount lost increasing from the ends toward the center. Hence, in a system containing an odd number of conjugated double bonds the central double bond has the smallest amount of double-bond character. In carotenoids this effect may possibly be enhanced by the structural difference between the central double bond and the other double bonds:



Because the activation energy for *trans* $\rightarrow$ *cis* isomerization about the central double bond is less than about the other double bonds, all members of a given set may be classified into "epimeric" pairs, the members of which are *cis* and *trans*, respectively, about the central double bond but otherwise possess the same configuration. The members of each pair are especially easily interconvertible, and the central-*trans*-isomer of each pair is more stable thermodynamically than its central-*cis*-isomer. For each pair the thermal isomerization



is expected to occur readily at lower temperatures than isomerizations involving other double bonds. Since the activation energies for forward and reverse reactions differ by the heat of the reaction, the less stable isomer of each pair (the central-*cis* compound) should isomerize at lower temperatures than the other member of the pair (116).

It remains to be seen how extensively such predictions can be used in practical experimentation. In any case, the above conclusions are in agreement with the observed appearance of a main isomer which is produced upon isomerization of an all-*trans* carotenoid and which possesses the highest *cis*-peak in the set.

Another use of thermal or photochemical methods is possible when a moderate heating or illumination yields mainly another member of the set. For example, if a mono-*cis* compound forms mainly a certain di-*cis* compound, it is reasonable to assume that the two pigments have one *cis*-position in common. An example to illustrate this method will be found in Part III under bixin (page 329).

2. Methods based on spectra

The spectroscopic phenomena which form the basis for the assignment of configurations take place partly in the visible and partly in the ultraviolet region.

(a) *Investigations in the visible region:* Since the visual maxima of all members of a stereoisomeric set are located at shorter wave lengths than the corresponding maxima of the all-*trans* compound, this difference, if correctly interpreted, should give us the number of the *cis* bonds in each case. The main difficulty lies in the lack of a reliable theoretical basis which would show the precise relation between this difference and the position of a *cis* double bond in the long unsaturated system. It may well be that the decrease in wave length caused by one *trans* $\rightarrow$ *cis* rotation is smaller for a peripheral double bond than for one situated at or near the center of the system. In practice, however, no essential differences of this character have been noticed up to the present time.

According to probability, when an all-*trans* compound undergoes isomerization the formation of mono-*cis* pigments will be favored. The main isomer (or one of the two main isomers) formed possesses a visually observed spectrum in petroleum ether or benzene in which the position of the longest wave-length maximum has been shifted $5\text{ m}\mu$ ($\pm 1\text{ m}\mu$) relative to the maximum of the corresponding all-*trans* compound. It is safe to assume that this regularly observed minimum difference is caused by one *trans* $\rightarrow$ *cis* rotation. Stereoisomers with about $10\text{ m}\mu$ difference should be classified as di-*cis* compounds, etc. On this basis, by using the values listed in table 21 (third column), it is possible to differentiate between all-*trans*, mono-*cis*, and di-*cis* members of a set, even if some doubtful cases may arise. If the spectral difference is considerably greater than $10\text{ m}\mu$, the insecurity increases, because nothing definite is known about the additivity of spectral differences and their dependence on the relative position of the double bonds concerned.

In principle, some configurations may also be derived from the relative intensities of the highest extinctions of each member of a set.

This principle has been used by Pauling (62), for example, in the case of lycopene and neolycopene. Pauling pointed out that the integrated absorption coefficients should be approximately proportional to the squares of the actual distances between the ends of the conjugated system. The all-*trans* member possesses the highest values in each set. An application of this principle will be given in Part III for methylbixin (p. 332).

(b) *Investigations in the cis-peak region:* The *cis*-peak effect was first observed in complicated stereoisomeric mixtures in which the unchanged portion of the all-*trans* compound amounted to the half or more of the total pigment. Consequently, the rest of the mixture, composed of a number of *cis* compounds, must show a higher molecular extinction value at the peak wave length than the entire equilibrium mixture.

A resolution of the effects can be conveniently carried out by chromatographic analysis of the mixture and the determination of the molecular extinction of each isomer at the *cis*-peak wave length. The first experiment of this kind, with Polgár (119), showed that those members of the β -carotene set which, on the Tswett column, are adsorbed below the all-*trans* pigment are responsible for the major fraction of the peak observed upon iodine catalysis. Further investigation has proved that generally one or two main isomers are responsible for the *cis*-peak observed in an isomerization mixture (table 20).

A characteristic constant of each stereoisomer is its "cis-peak effect," i.e., the difference between its molar extinction and that of the all-*trans* form at the *cis*-peak wave length (table 21, fifth column, p. 312).

In the following we intend to give a stereochemical survey of some carotenoid sets. The author does not claim that the difficult problem of the assignment of configurations has reached a final solution. However, the phenomena observed permit a reasonable stereochemical discussion in several cases for which formerly no experimental data whatsoever were available.

It will be practical to consider separately the carotenoid sets which contain an odd member of conjugated double bonds and those which contain an even number of such bonds.

The stereochemistry of the former subclass is simpler, especially if the molecule is symmetrical (e.g., β -carotene). In this case, we can speak of a truly "central" double bond. For any non-central double bond of the system, there

TABLE 20

Stereoisomers responsible for the major part of the cis-peak effect of the total equilibrium mixture obtained by iodine catalysis

STEREoisomERIC SET	MEMBER OF THE SET	APPROXIMATE PERCENTAGE OF THE MEMBER IN THE EQUILIBRIUM	APPROXIMATE PERCENTAGE OF THE TOTAL <i>cis</i> -PEAK EFFECT CAUSED BY THE MEMBER	REFERENCES
α -Carotene.....	Neo B	13	55	(120)
β -Carotene.....	Neo B	25	75	(65)
Lycopene.....	Neo A	30-40	95	(128)
Lutein.....	Neo A	17	70	(128)
Cryptoxanthin.. . . .	Neo A	23	60	(113)
Zeaxanthin.....	Neo A + Neo B	30	90	(106)
Capsanthin.....	Neo A	20	80	(105)

is another which is stereochemically equivalent. If n sterically effective double bonds are present, $(n - 1)/2 + 1$ mono-*cis* isomers are expected.

The situation is more complicated if the number of conjugated double bonds is even (e.g., α -carotene). In this case, the center of the chromophore is a single bond and there are two "central" double bonds. Considering the structure of carotenoids, the system is then non-symmetrical, and the expected number of mono-*cis* compounds is equal to that of the sterically effective double bonds.

3. Stereochemical sets with an odd number of conjugated carbon-carbon double bonds

(a) Lycopene set (figures 16, 17, 19-21) (116, 120)

The number of possible stereoisomers is seventy-two. The chromophore is structurally and stereochemically symmetrical and contains seven effective double bonds of which 1 and 11, 3 and 9, and 5 and 7 have equivalent positions. This set was used by Pauling and our group to develop a theory of the *cis*-peak effect.

TABLE 21

*Typical spectroscopic data and the cis-peak effect in some stereochemical sets of carotenoids
(in the sequence of decreasing adsorption affinities within the sets)*

STEREoisomeric SET	MEMBER OF THE SET	DIFFERENCE BETWEEN THE VISUALLY ESTABLISHED LONGEST WAVE- LENGTH MAXI- MUM OF THE MEMBER AND THAT OF THE ALL- <i>trans</i> FORM (IN PETROLEUM ETHER)	MOLECULAR EXTINCTION COEFFICIENT AT THE <i>cis</i> - PEAK WAVE LENGTH $E_{1\text{cm.}} \times 10^{-4}$	DIFFERENCE OF $E_{1\text{cm.}} \times 10^{-4}$ FOR MEMBER OF THE SET AND ALL- <i>trans</i> FORM AT THE <i>cis</i> -PEAK WAVE LENGTH	REFERENCES
		$m\mu$			
α -Carotene (in hexane).....	Neo U	5.5	1.2	0.4	(120)
	Neo V	11.5	1.1	0.3	
	Neo W	6.5	1.6	0.8	
	Neo X	13.5	2.7	1.9	
	All- <i>trans</i>	0	0.8	0	
	Neo A	8.5	3.8	3.0	
	Neo B	10.5	3.8	3.0	
	Neo C	4.5	4.5	3.7	
β -Carotene (in hexane).....	Neo U	5	1.3	0.5	(120)
	Neo V	13.5	0.8	0	
	All- <i>trans</i>	0	0.8	0	
	Neo B	10.5	3.4	2.6	
	Neo E	8.5	3.4	2.6	
γ -Carotene (in hexane).....	All- <i>trans</i>	0	0.95	0	(116)
	Neo- γ -carotene	5			
	Pro- γ -carotene	31	1.3	0.35	
Lycopene (in hexane).....	All- <i>trans</i>	0	1.4	0	(116, 120)
	Neo A	5	6.8	5.4	
	Neo B	8	3.7	2.3	
	Unnamed crys- taline isomer	28	1.3	-0.1	
	Prolycopene	34	1.6	0.2	
	All- <i>cis</i>	38.5	2.2	0.8	
Cryptoxanthin (in hexane)	Neo U	5	1.5	0.3	(113)
	All- <i>trans</i>	0	1.2	0	
	Neo A	6.5	4.2	3.0	
	Neo B	4.5	4.5	3.3	
Cryptoxanthin (in benzene).....	*				(113)
	Neo U	5	1.7	0.7	
	All- <i>trans</i>	0	1.0	0.7	
	Neo A	6.5	3.4	2.4	
	Neo B	4.5	4.6	3.6	

TABLE 21—Continued

STEREISOOMIC SET	MEMBER OF THE SET	DIFFERENCE BETWEEN THE VISUALLY ESTABLISHED LONGEST WAVELENGTH MAXIMUM OF THE MEMBER AND THAT OF THE ALL- <i>trans</i> FORM (IN PETROLEUM ETHER)	MOLECULAR EXTINCTION COEFFICIENT AT THE <i>cis</i> -PEAK WAVELENGTH $E_{1\text{cm.}} \times 10^{-4}$	DIFFERENCE OF $E_{1\text{cm.}} \times 10^{-4}$ FOR MEMBER OF THE SET AND ALL- <i>trans</i> FORM AT THE <i>cis</i> -PEAK WAVELENGTH	REFERENCES
		$m\mu$			
Zeaxanthin (in benzene)	Neo A	5.5	4.4	3.7	(113)
	Neo B	5.5	2.4	1.7	
	Neo C	8.5	3.9	3.2	
	All- <i>trans</i>	0	0.7	0	
Lutein (in benzene) .	Neo A	6	4.9	4.1	(120)
	Neo B	7	2.1	1.3	
	All- <i>trans</i>	0	0.8	0	
Capsanthin (in benzene)	Neo A	6	4.4	3.4	(67)
	Neo B	6	2.7	1.6	
	Neo C	10.5	1.9	0.8	
	All- <i>trans</i>	0	1.1	0	

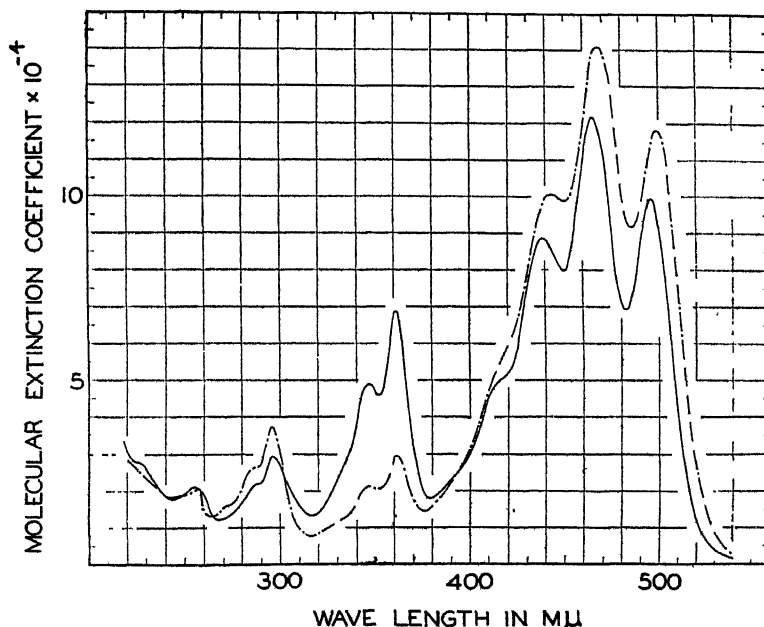


Fig. 19. Molecular extinction curves of neolycopene A in hexane: —, fresh solution of neolycopene A; ---, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 65. 1944 (1943))

The main isomer observed in chromatograms is neolycopene A, which has the highest *cis*-peak so far measured in any set. On the basis of this characteristic and the spectral difference of $4.5\text{ m}\mu$ (table 21), this isomer must be δ -mono-*cis*-lycopene. Neolycopene B is a di-*cis* isomer which may well be 1,6- or 3,6-di-*cis*-lycopene. It has been mentioned earlier that polycopene is very probably an all-but-one-*cis* isomer. We assume that its correct designation is 1,3,5,7,9,11-hexa-*cis*-lycopene. Its relatively high thermostability is explained by the effect of the many peripheral *cis* double bonds which decrease the conjugation

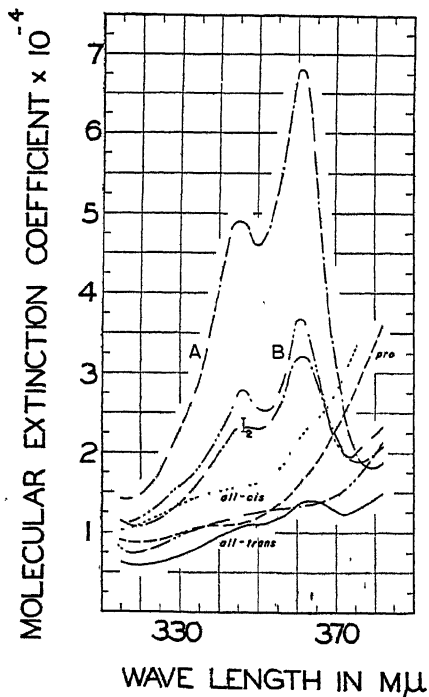


FIG. 20. Molecular extinction curves of some members of the stereoisomeric lycopene set in the *cis*-peak region in hexane: ····· denotes an unnamed crystalline isomer; I_2 indicates the equilibrium mixture obtained upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 141 (1944))

and stabilize the centrally located *trans* double bond, the only stereochemically effective double bond which remained in *trans* configuration. All-*cis*-lycopene should be termed 1,3,5,6,7,9,11-hepta-*cis*-lycopene.

(b) β -Carotene set (figures 2, 22, 23) (120)

The number of possible stereoisomers is twenty. The chromophore is structurally and stereochemically symmetrical and contains five effective double bonds, of which 3 and 9 as well as 5 and 7 have equivalent positions. All stereoisomers of this set are represented in figure 2 (on p. 279) by their carbon skeletons.

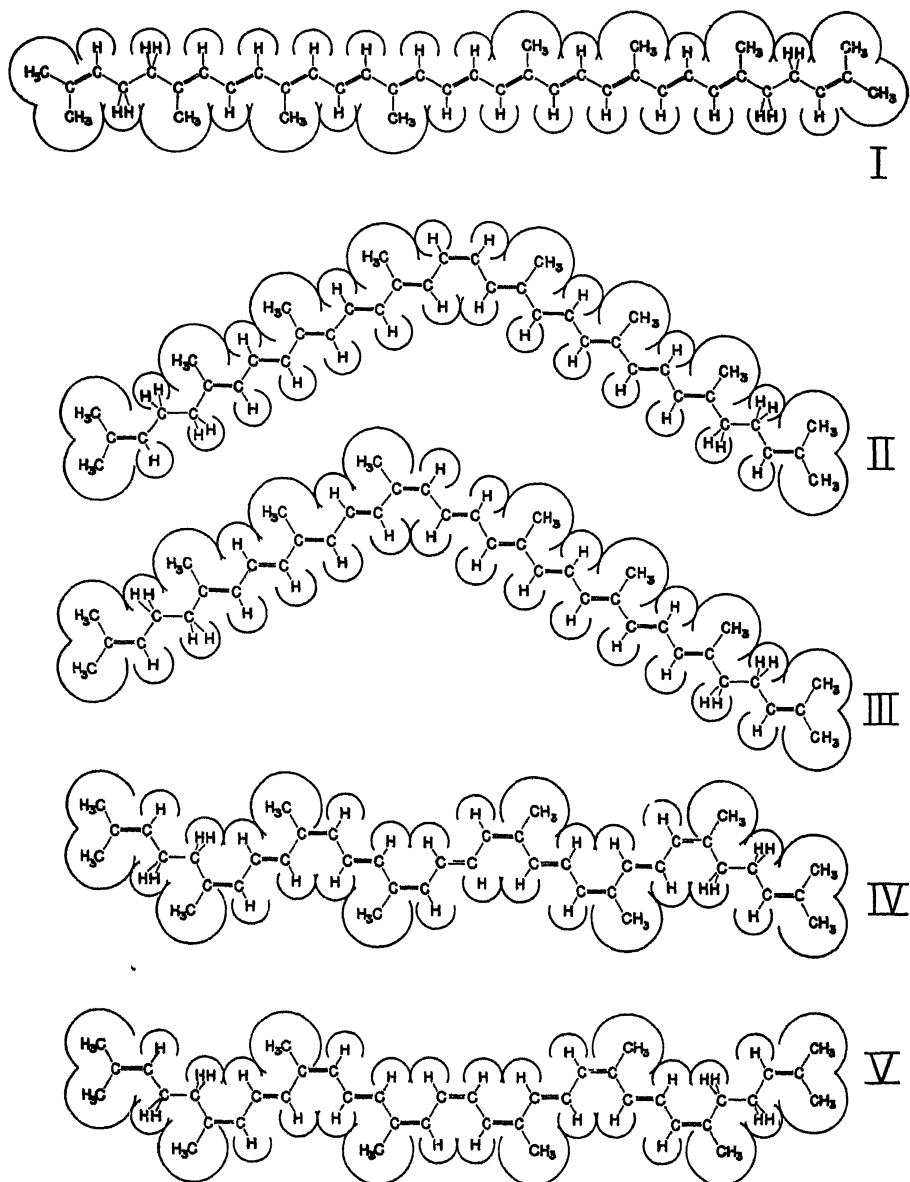


FIG. 21. Suggested stereochemical structures of some members of the lycopene set: I, all-*trans*-lycopene; II, neolycopene A; III, δ -*cis*-lycopene; IV, polycopene; V, all-*cis*-lycopene. (From J. Am. Chem. Soc. 65, 1946 (1943))

The positions of the visual maxima show that neo- β -carotene U is a mono-*cis*, and neo V a di-*cis* compound. Neither can contain a δ -*cis* central double bond because of their low *cis*-peaks and relatively high thermostability when refluxed.

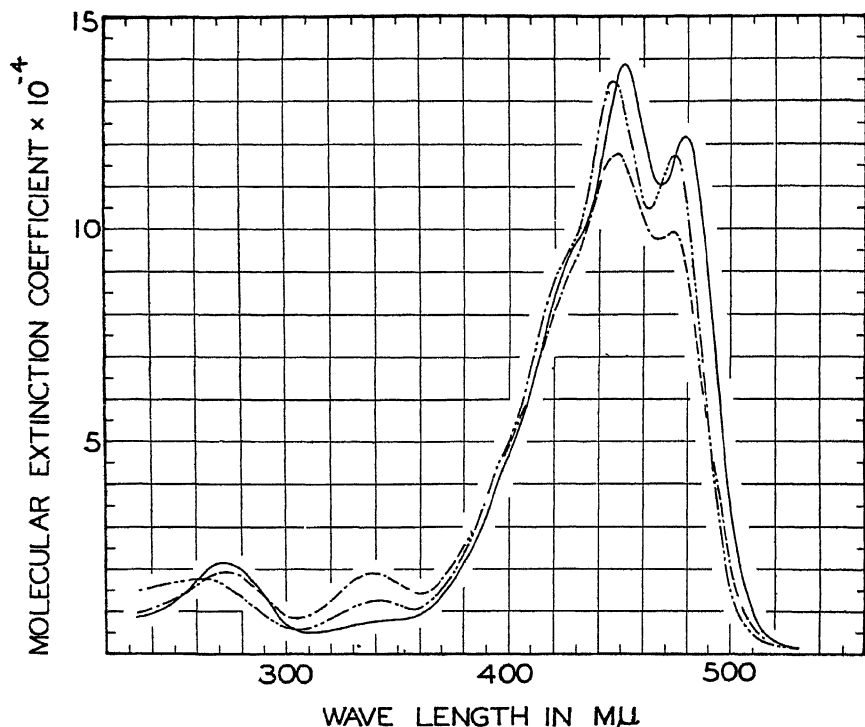


FIG. 22. Molecular extinction curves of neo- β -carotene U, compared with that of all-*trans*- β -carotene, in hexane: —, β -carotene; — · —, fresh solution of neo- β -carotene U; · · · · ·, mixture of stereoisomers obtained from neo U upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **65**, 1524 (1943))

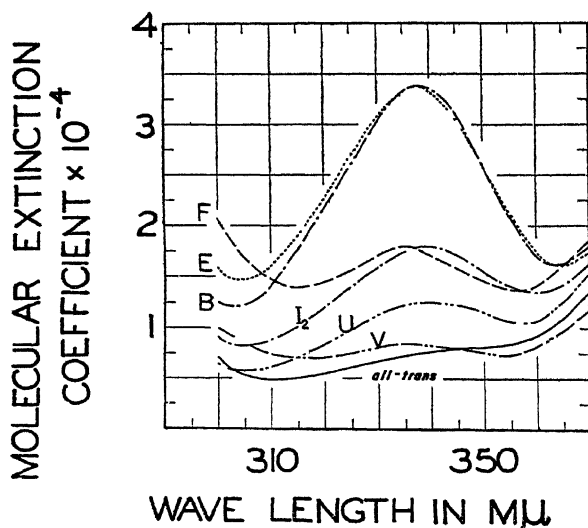


FIG. 23. Molecular extinction curves of some members of the stereoisomeric β -carotene set in the *cis*-peak region in hexane. I_2 indicates the equilibrium mixture obtained upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **66**, 139 (1944))

The most probable configurations are: neo- β -carotene U = 3-mono-*cis*- β -carotene, and neo- β -carotene V = 3,9-di-*cis*- β -carotene. However, a 5,7-di-*cis* configuration cannot be excluded in the latter case.

A main isomer observed in chromatograms, neo- β -carotene B, can be interpreted as a di-*cis*- β -carotene which has one of its *cis*-bonds in the central position; such an assignment is substantiated by the high *cis*-peak and the relatively slight thermostability. Since a comparison of the *cis*-peak with the models excludes the 5,6-di-*cis* configuration, the best assumption is that neo- β -carotene B is

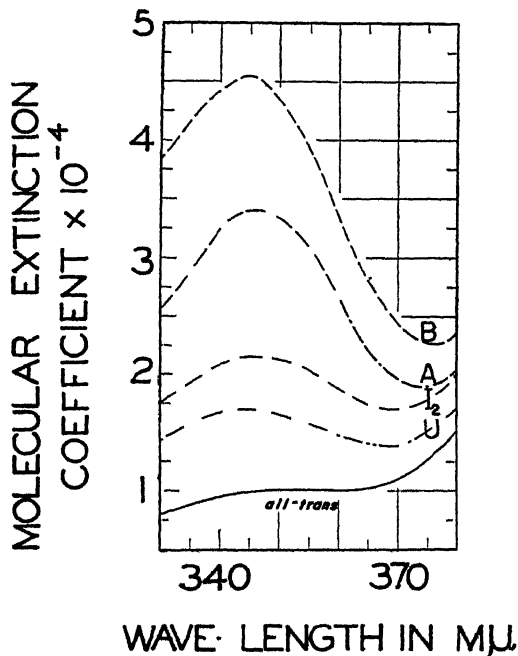


FIG. 24. Molecular extinction curves of some members of the stereoisomeric cryptoxanthin set in the *cis*-peak region in benzene. I₂ indicates the equilibrium mixture obtained upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **66**, 320 (1944))

3,6-di-*cis*- β -carotene. The minor isomer, neo E, seems to belong to the same type, but because of its lability no definite spatial structure has been suggested.

(c) Cryptoxanthin set (figure 24) (113)

The number of possible stereoisomers is thirty-two. The chromophore is stereochemically symmetrical but structurally unsymmetrical because of the presence of a hydroxyl group at only one end of the molecule. All five effective double bonds, *viz.*, 3,5,6,7, and 9, may give rise to different mono-*cis* isomers.

Evidently, the neo forms U and B are mono-*cis* compounds. The enormous difference in their *cis*-peaks suggests that neocryptoxanthin U is 3- or 9-mono-*cis*-cryptoxanthin and neocryptoxanthin B is 6-mono-*cis*-cryptoxanthin. The

configuration of neo A is somewhat uncertain because its relatively high *cis*-peak and the spectral difference of $6.5\text{ m}\mu$ permit several interpretations. This isomer could be a di-*cis* compound with one central and one peripheral double bond in *cis* configuration.

(d) Zeaxanthin set (figure 25) (113)

The general characteristics are the same as given for the β -carotene set.

The best interpretation for the two main isomers, which cannot be differentiated in the visual spectroscope but which have different *cis*-peaks, is: neo-zeaxanthin A = δ -mono-*cis*-zeaxanthin and neozeaxanthin B = δ -mono-*cis*-zeaxanthin. The peak of the minor isomer C is very high, and its spectral

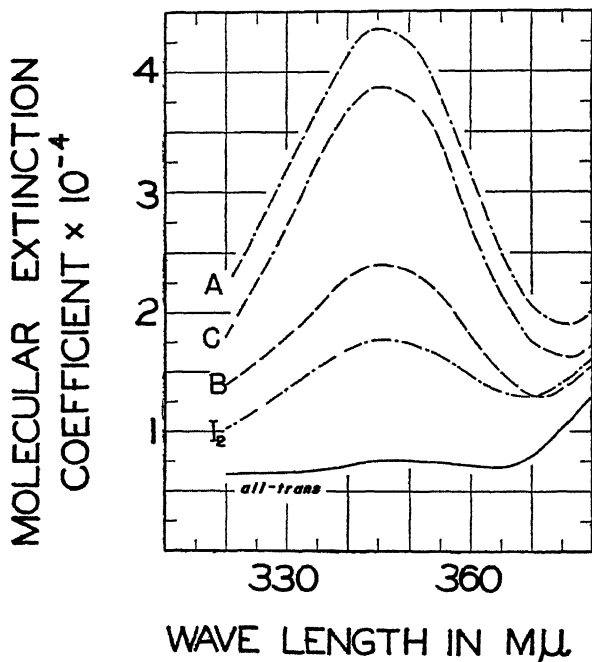


FIG. 25. Molecular extinction curves of some members of the stereoisomeric zeaxanthin set in the *cis*-peak region in benzene. I_2 indicates the equilibrium mixture obtained upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **66**, 320 (1944))

difference in the visible region is $8.5\text{ m}\mu$. This isomer could be a di-*cis* compound with one *cis* double bond in the center, *viz.*, 3,6-di-*cis*-zeaxanthin.

(e) γ -Carotene set (figures 5, 8, 18) (116)

The number of possible stereoisomers is sixty-four. The chromophore is stereochemically symmetrical but structurally unsymmetrical because of the presence of one cyclic and one acyclic terminal group. All six effective double bonds, *viz.*, 1,3,5,6,7, and 9, may give rise to different mono-*cis*-isomers.

This set has not yet been investigated in detail. The observed main fraction, "neo- γ -carotene", has a spectral difference of $5\text{ m}\mu$ and must contain mainly

a mono-*cis* compound. Pro- γ -carotene is probably 3,5,7,9,11-penta-*cis*- γ -carotene.

4. Stereochemical sets with an even number of conjugated carbon-carbon double bonds

(a) α -Carotene set (figures 3, 26, 27) (120)

The number of possible stereoisomers is thirty-two. The chromophore is structurally unsymmetrical because of the different positions of the double

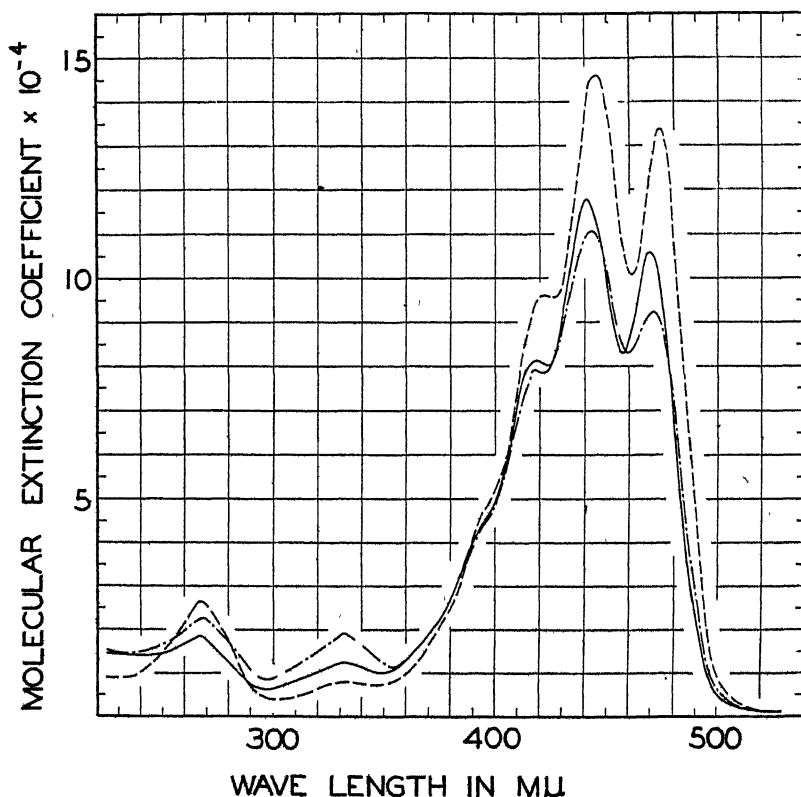


FIG. 26. Molecular extinction curves of neo- α -carotene U, compared with that of all-*trans*- α -carotene, in hexane: — — —, all-*trans*- α -carotene; —, fresh solution of neo- α -carotene U; — · —, mixture of stereoisomers obtained from neo U upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 142 (1944))

bonds in the rings and also stereochemically unsymmetrical because of the even number of conjugated double bonds. That the two "central" double bonds, 5 and 6, are structurally different follows from the position of the methyl side chains. The effective double bonds are 3,5,6,7, and 9. Considering these features, five mono-*cis*, ten di-*cis*, ten tri-*cis*, five tetra-*cis*, and one penta-*cis* members of the α -carotene set can be expected (cf. p. 280).

The neo- α -carotenes U, V, and W show considerable thermostability and very moderate *cis*-peaks. The isomers U and W seem to contain one *cis* double bond, whereas V must be reasonably interpreted as a di-*cis* compound. The most probable configurations are: neo U = 9-mono-*cis*- α -carotene; neo W = 3-mono-*cis*- α -carotene; and neo V = 3,9-di-*cis*- α -carotene. A di-*cis* compound, neo X, was preponderantly formed upon mild heating of neo U solutions; therefore one of its *cis* double bonds, *viz.* 9, will probably have a position identical with that of neo U. Since the 5,9 and 6,9 configurations must be assigned to other isomers (see below), neo- α -carotene X is probably 7,9-di-*cis*- α -carotene.

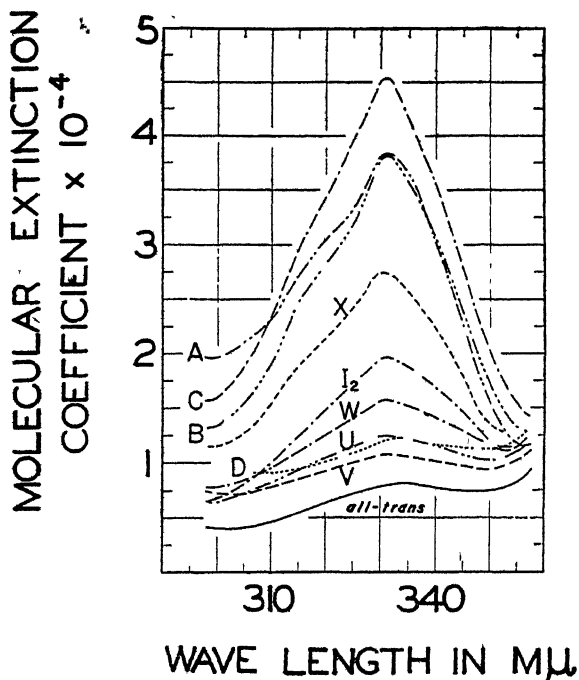


FIG. 27. Molecular extinction curves of some members of the stereoisomeric α -carotene set in the *cis*-peak region in hexane. I_2 indicates the equilibrium mixture obtained upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 140 (1944))

Neo C, a labile minor isomer, could be 6- or 5-mono-*cis*- α -carotene. One of the main observed isomers, neo B, has approximately the same *cis*-peak, and in the visible region the same spectral difference from the all-*trans* maximum as neo- β -carotene B. Its high peak suggests that one of its *cis* positions is central and the other peripheral, because neither two peripheral nor two central *cis* double bonds would explain the height of the peak observed. The non-equivalence of the 5- and 6-positions of the models shows that neo- α -carotene B may be either 5,9- or 6,9-di-*cis*- α -carotene. Since the neo forms A and B have practically identical peaks, it is reasonable to say that for these two di-*cis* isomers the following four configurations are possible: 5,9; 6,9; 3,5; and 3,6.

(b) Lutein set (figure 28) (120)

The characteristics of this dihydroxy- α -carotene set are identical with those of α -carotene. Only two main observed isomers, neo A and B, have been investigated. Neolutein A is very probably δ - or 5 -mono-*cis*-lutein. The extinction at the *cis*-peak of neo B is less than the half of that of neo A. The difficulty in assigning a definite configuration to neo B lies in the fact that the visual spectra of A and B differ only by 1 $m\mu$. Neolutein B could be either β -mono-*cis*- or θ -mono-*cis*-lutein.

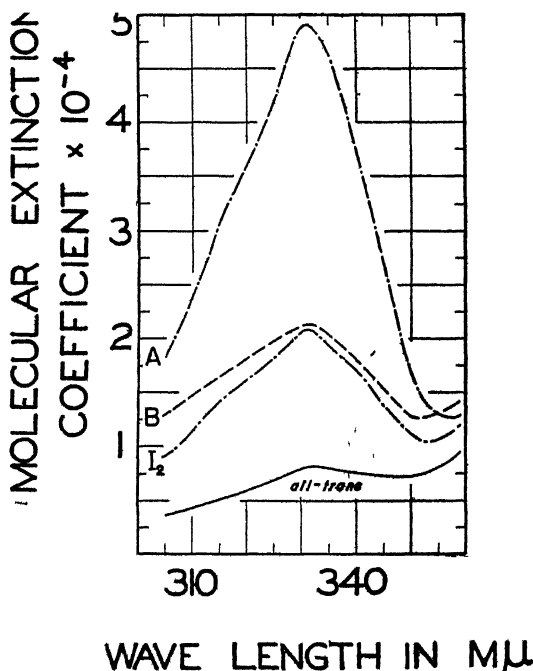


FIG. 28. Molecular extinction curves of some members of the stereoisomeric lutein set in the *cis*-peak region in hexane. I_2 indicates the equilibrium mixture obtained upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. **66**, 140 (1944))

(c) Capsanthin set (figure 29) (67)

The number of possible stereoisomers is thirty-two. This set contains ten conjugated carbon-carbon double bonds in conjugation with one carbonyl group. The chromophore is structurally and stereochemically unsymmetrical. The five effective double bonds are $\beta, 5, 6, 7$, and 9 .

The two main isomers, neo A and B, are evidently mono-*cis* compounds, as shown by the identical position of their visually observed maxima. On the basis of the high *cis*-peak of A and the considerable peak of B the most probable configurations are: neocapsanthin A = δ -mono-*cis*-capsanthin and neocapsanthin B = 5 -mono-*cis*- or 7 -mono-*cis*-capsanthin. The positions of the two *cis*

double bonds in the minor isomer neocapsanthin C cannot be stated definitely; the models do not exclude the possibility that one of its *cis* positions is at or near the center.

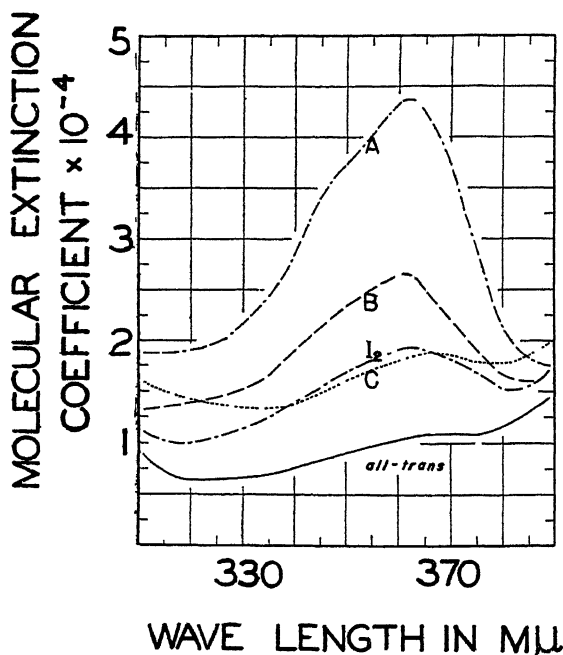
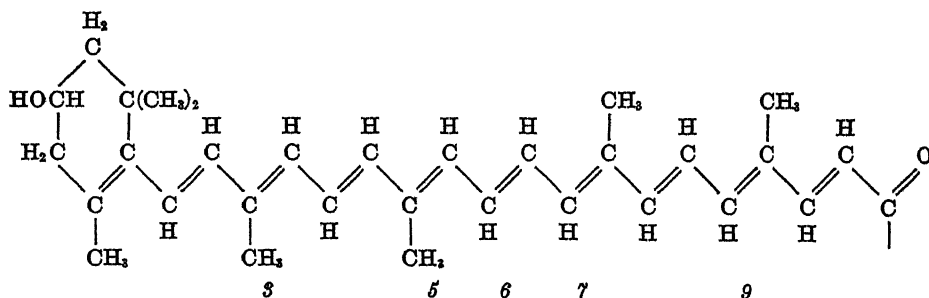


FIG. 29. Molecular extinction curves of some members of the stereoisomeric capsanthin set in the *cis*-peak region in benzene. I_2 indicates the equilibrium mixture obtained upon iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 189 (1944))



The chromophore of capsanthin
(The sterically effective double bonds are numbered)

III. CAROTENOIDS CONTAINING LESS THAN FORTY CARBON ATOMS

Although the quantity of the natural pigments belonging to this class is negligible compared with the amounts of C_{40} -carotenoids which are synthesized in the vegetable tissue each year, these pigments are, nevertheless, important from

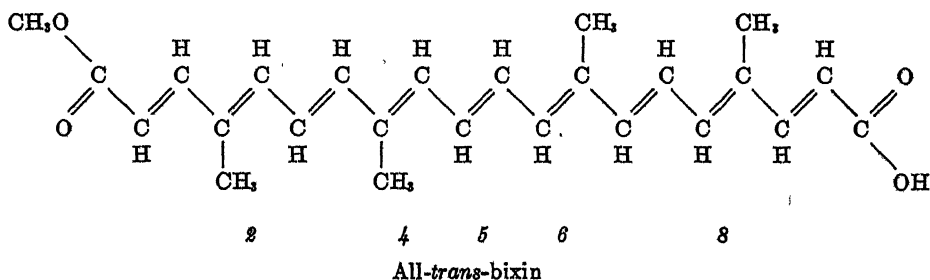
the stereochemical viewpoint. Indeed, some of the fundamental observations in this field were made with bixin and crocetin at a time when nothing was known of stereoisomeric polyenes with forty carbon atoms.

Bixin, $C_{25}H_{30}O_4$, occurs in the seed hulls of the tropical Annatto tree, *Bixa orellana* L., and constitutes the main pigment of the industrial annatto or Orlean. A pigment of still lower molecular weight, crocetin, $C_{20}H_{24}O_4$, is present in the stigma of saffron (*Crocus sativus*) in the form of the gentiobioside, crocin. Certain other flowers also contain crocetin.

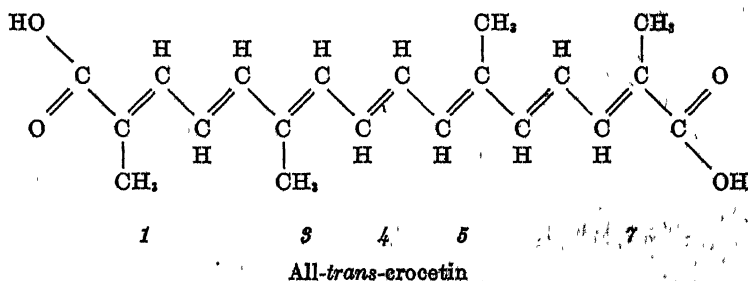
The formulas show the presence of nine and seven conjugated carbon-carbon double bonds in conjugation with carboxyl groups. As can be seen from the characteristic position of the methyl side chains, the chromophores are identical with the middle section of the C_{40} -carotenoids. The correctness of the symmetrical formulas was proved by Karrer *et al.* (21).

The bixin molecule contains five stereochemically effective double bonds. Considering its non-symmetrical structure, the calculated number of stereoisomers is thirty-two (as for α -carotene), whereas in the case of the free carboxylic acid, norbixin, or its dimethyl ester, methylbixin, this number is reduced to twenty (as for β -carotene).

Crocetin constitutes a similar case with five effective double bonds. The number of possible stereoisomers is twenty for crocetin or dimethylcrocetin but thirty-two for methylcrocetin.



Monomethyl ester of 3,7,12,16-tetramethyloctadecanonaene-1,18-dicarboxylic acid (the stereochemically effective double bonds are numbered in *italics*); the dimethyl ester is termed methylbixin, and the free dicarboxylic acid, norbixin



1,5,10,14-Tetramethyl-tetradecaheptaene-1,14-dicarboxylic acid, (the stereochemically effective double bonds are numbered in *italics*); the methyl esters are termed methylcrocetin and dimethylcrocetin

A. BIXIN

The history of bixin stereochemistry reaches as far back as 1923, when Herzig and Faltis (17), in a single, unreproducible experiment, obtained from Orlean, instead of the well-known bixin ("labile bixin"), an isomer, " β -bixin" ("stable bixin"), with higher melting point, greater stability, and longer wave-length spectrum. The same starting material gave ordinary bixin in other experiments. An analogous observation was reported later by Karrer, Helfenstein, Widmer, and van Itallie (21). These investigators were the first to express the opinion that the two bixins are in a *cis-trans* relationship (*cf.* 31), a fact which was proved by the conversion of ordinary bixin into the β -bixin by means of iodine. If iodine is added to an ethyl acetate solution of methylbixin, the methyl ester of the β -bixin crystallizes out on short standing. According to Kuhn and Winterstein (38), catalytic amounts of the halogen are sufficient to bring about this conversion. Perbenzoic acid in chloroform is also effective (88).

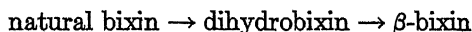
The structural identity of the two bixins, in spite of spectroscopic differences (table 22), was proved chemically by Kuhn and Winterstein (38) and Kuhn and

TABLE 22

Visually observed spectral maxima of some members of the methylbixin set, listed in the sequence of decreasing adsorption affinities

MEMBER OF THE SET	IN PETROLEUM ETHER (B.P. 60-70°C.)		IN BENZENE	
	$m\mu$	$m\mu$	$m\mu$	$m\mu$
Natural methylbixin.....	485	453.5	503	470
All- <i>trans</i> -methylbixin.....	490	457	508.5	475
Neomethylbixin A.....	485	454	502.5	469
Neomethylbixin B.....	471	444.5	491	458
Neomethylbixin C.....	479.5	449	496	463

Drumm (29, 30). When two hydrogen atoms were added to either bixin by treatment with zinc and glacial acetic acid or with titanium trichloride and ammonia (88), the same dihydro product appeared in both experiments. This is geometrically understandable in the case of a Thiele addition of two hydrogen atoms to *cis-trans* isomers. By oxidation in air, in the presence of pyridine, dihydrobixin formed that bixin which can be obtained from natural bixin by iodine catalysis. The total transition is:



In the following cases a chemical change produced a direct transition from "labile" into "stable" compound: labile methylbixin when saponified with aqueous-alcoholic alkali gave stable norbixin; alkali converted labile methylbixin (to a small extent) into stable bixin; and labile norbixin when methylated with methanolic hydrogen chloride gave stable methylbixin instead of the expected labile form, which can be obtained by the action of diazomethane or dimethyl sulfate on norbixin (for details see 102). An "isobixin" which had been

described by van Hasselt (14) could not be reproduced by Karrer and Takahashi (23); its homogeneity is doubtful.

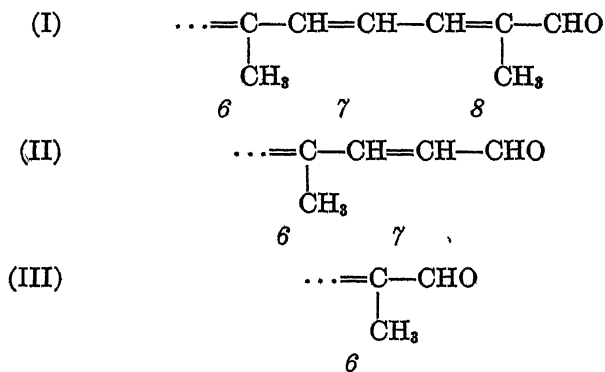
When all these observations are considered, natural bixin must contain at least one *cis* double bond in a position where the artifact is *trans*. This also causes a difference in adsorbability, as pointed out in a brief remark of Winterstein and Stein (99).

The nomenclature which has been employed so far includes the following designations for the two bixins and analogous terms for the two methylbixins and norbixins:

- (a) Ordinary bixin = natural bixin = *cis*-bixin = α -bixin = labile bixin = bixin II = lower melting bixin (m.p. 198°C. (corr.))
- (b) Isobixin = *trans*-bixin = β -bixin = stable bixin = bixin I = higher melting bixin (m.p. 220°C. (corr.))

"Stable" bixin is undoubtedly the all-*trans* member of the set.

Neither the number nor the position of the *cis* double bonds in the naturally occurring "labile" pigment had been determined in the investigations discussed above. So far as we know, the only approach to a solution of this problem was made by Karrer and Solmssen (22), who cautiously oxidized the two bixins with permanganate. In both cases the aldehydes I, II, and III, which evidently arose through a stepwise splitting of the chain near the free carboxyl group in bixin were observed:



The aldehydes I and II, when prepared by oxidation of stable bixin, were the *trans* forms of those obtained from labile bixin, but an identical all-*trans* aldehyde III resulted from both bixins. The authors, therefore, concluded (with reservation) that the double bond 7 has *cis* configuration in natural bixin. Although this conclusion must now be revised because bond 7 is a sterically hindered double bond, the remarkable method reported by Karrer and Solmssen may become useful in the future.

A recent re-investigation of the sterically simpler methylbixin set, in collaboration with Escue (111), gave the following results:

After isomerization and development with benzene-petroleum ether mixtures on calcium carbonate columns, in addition to the two well-known methylbixin isomers, two others, neo A and C, were isolated in crystalline form. Some characteristics of a fifth minor isomer, neo B, were determined in solution and a few other isomers occurring in traces were observed occasionally. Since "labile" methylbixin is much less labile than some other members of the set, it has been renamed "natural methylbixin", a name which implies that it has the same

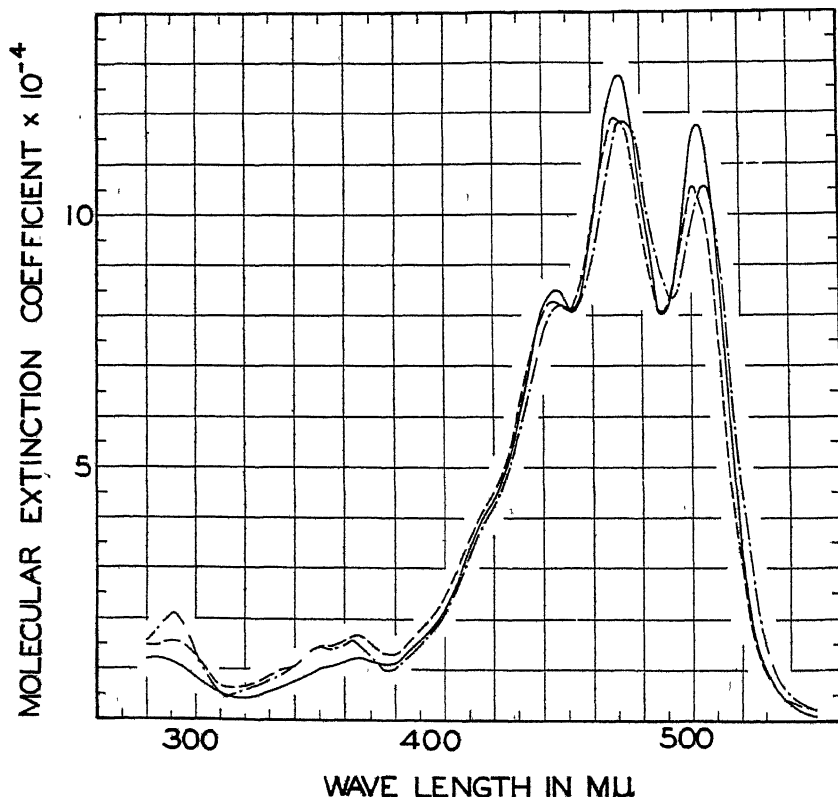


FIG. 30. Molecular extinction curves of natural methylbixin in benzene: —, fresh solution of natural methylbixin; — — —, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 323 (1944))

configuration as the bixin occurring in the *Bixa* seeds. Stable or *trans*-methylbixin is now termed "all-*trans*-methylbixin." Characteristic spectral data are given in table 22, in which the isomers are listed in the sequence of decreasing adsorption affinity. Upon addition of iodine the isomers shift their maxima to the following wave lengths; in petroleum ether, 488, 455 mμ; and in benzene, 506.5, 472 mμ.

The extent of spontaneous isomerization of natural methylbixin in benzene

at room temperature is about 3 per cent within 24 hr. After refluxing each pure isomer for 1 hr. the extent of stereoisomerization was: natural methylbixin, 26 per cent (mainly neo C formed); all-*trans*-methylbixin, 37 per cent (mainly neo C); neomethylbixin A, 68 per cent (mainly all-*trans*); and neomethylbixin C, 54 per cent (mainly natural methylbixin formed).

Extensive *cis-trans* rotations occur also when the crystals are melted and kept at 160°C. for 1 min. The composition of the iodine equilibrium mixture

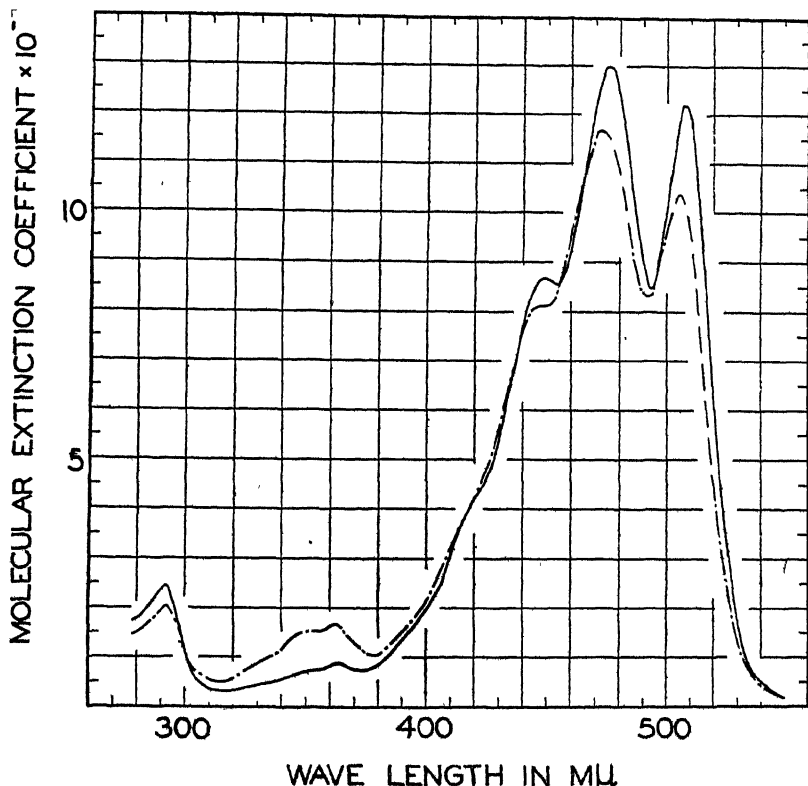


Fig. 31. Molecular extinction curves of all-*trans*-methylbixin in benzene: —, fresh solution of the all-*trans* compound; ---, after iodine catalysis at room temperature in light. The second curve is practically identical with that obtained after refluxing for 45 min. in darkness. (From J. Am. Chem. Soc. 66, 323 (1944))

is (with some variations): all-*trans*:neo A:neo C = 68:22:10. The configuration of natural methylbixin is so sensitive to iodine that this isomer practically disappears when the catalyst is added to its solution.

Extinction curves for bixins have been published by Smakula and by Karrer and Würzler (23a, 73). In figures 30 to 33 we give such curves for the four crystalline isomers, as well as for the stereoisomeric mixtures obtained upon refluxing or iodine catalysis. The changes observed upon such treatment are analogous

to those which take place with C_{40} -carotenoids. As illustrated by figure 34, light is needed for the development of the *cis*-peak in the presence of iodine. The *cis*-peak (like that of lycopene) possesses a characteristic fine structure, the cause of which is unknown.

With the exception of the all-*trans* form, the isomers studied are light sensitive, especially the neomethylbixins A and C. Under the conditions applied, no

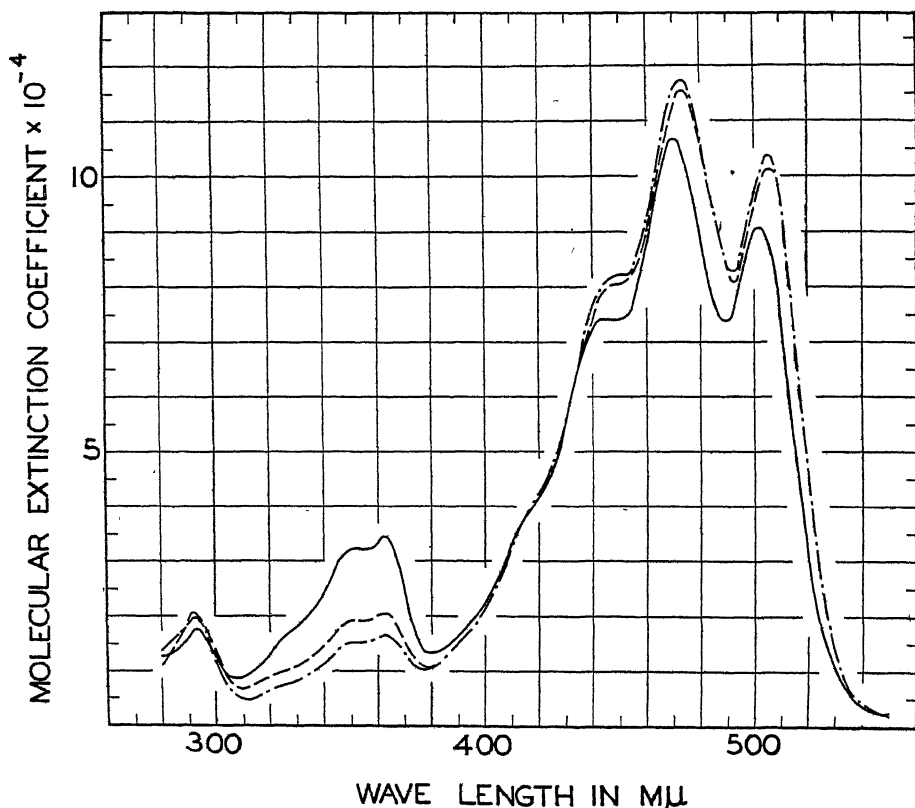


FIG. 32. Molecular extinction curves of neomethylbixin A in benzene: —, fresh solution of neomethylbixin A; ---, mixture of stereoisomers after refluxing in darkness for 45 min.; —·—, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 324 (1944))

irreversible conversion or bleaching occurred during the exposure to sunshine, the effect of which is demonstrated by figures 35 and 36 (p. 330).

Assignment of configurations

Let us consider now the configurations. On the basis of the spectral differences from the all-*trans* compound in the visual region (table 22), natural methylbixin and neo A are doubtlessly mono-*cis* compounds but neo C is a di-*cis*- and B a tri-*cis* (or possibly tetra-*cis*)-methylbixin. It is a peculiar feature of this set that upon refluxing or insolation of natural methylbixin practically no all-*trans*

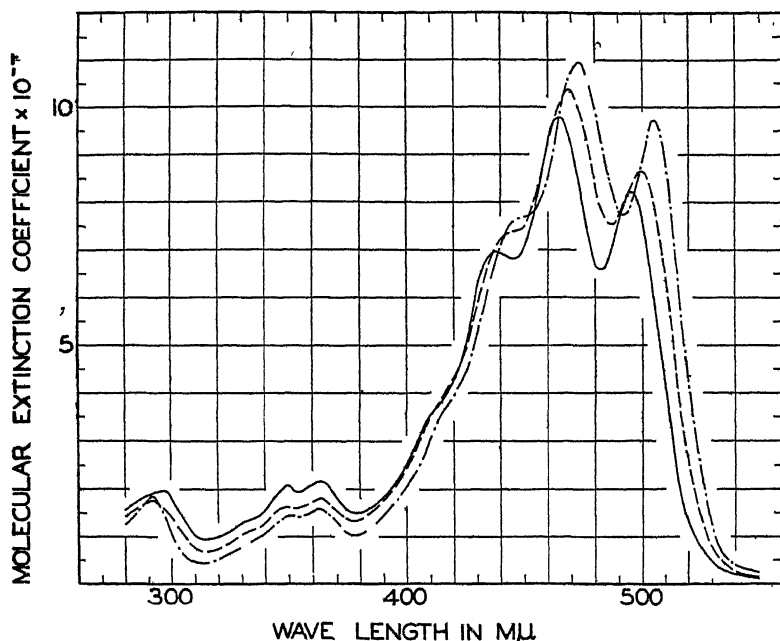


FIG. 33. Molecular extinction curves of neomethylbixin C in benzene: —, fresh solution of neomethylbixin C; — — —, mixture of stereoisomers after refluxing in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From J. Am. Chem. Soc. 66, 325 (1944))

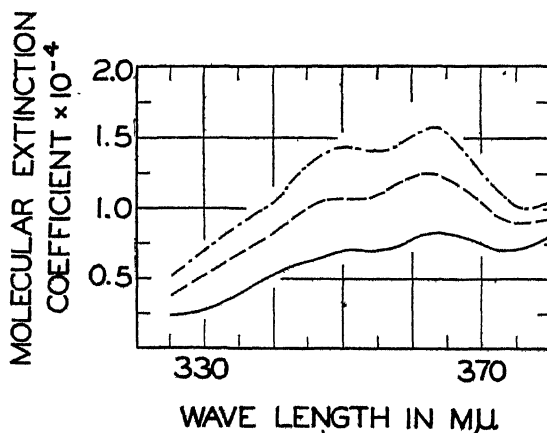


FIG. 34. Influence of illumination on the development of the *cis*-peak in an iodine-catalyzed solution of all-*trans*-methylbixin in benzene: —, molecular extinction curve after standing in darkness with iodine for 30 min.; — — —, molecular extinction curve after 5 sec. illumination; — · —, molecular extinction curve after 30 sec. illumination. (From J. Am. Chem. Soc. 66, 325 (1944))

form appears, and *vice versa*. In the absence of catalysts the stability of the *cis* double bond in natural methylbixin is so great that the molecule undergoes a

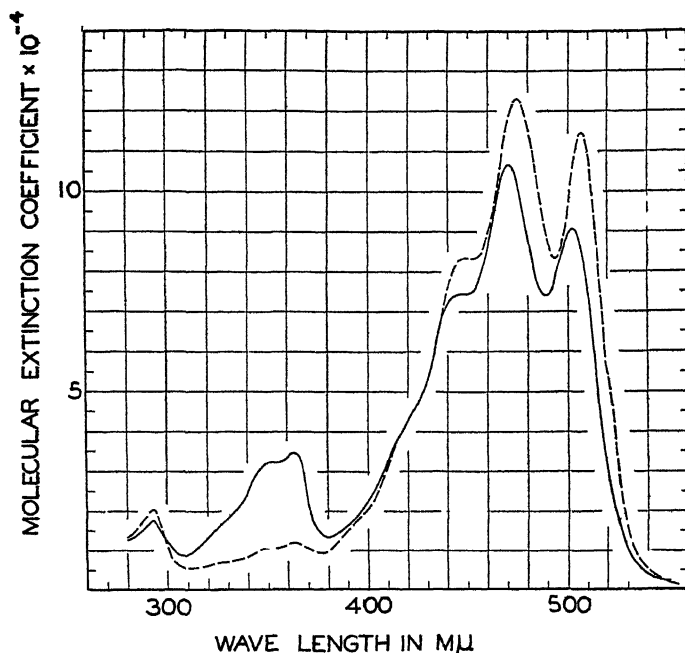


FIG. 35. Photo-isomerization of neomethylbixin A in benzene: —, fresh solution of neomethylbixin A; — — —, mixture of stereoisomers after insolation for 15 min. (From J. Am. Chem. Soc. **66**, 326 (1944))

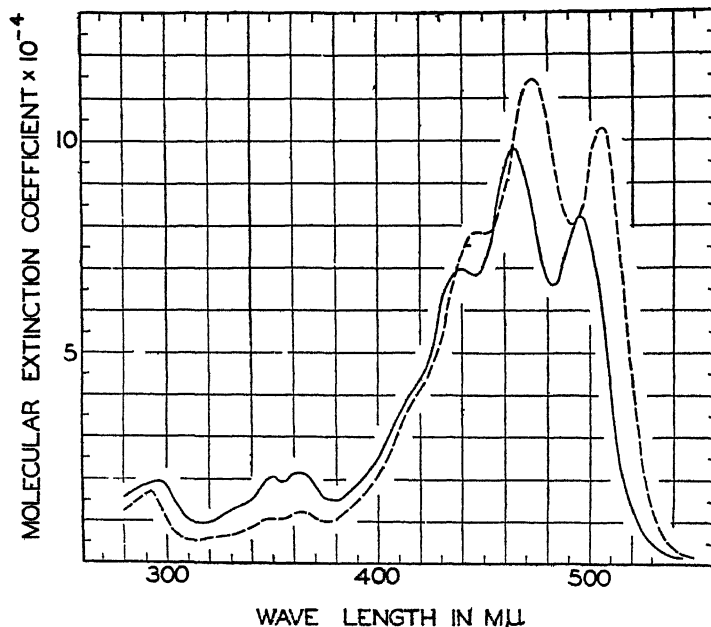


FIG. 36. Photo-isomerization of neomethylbixin C in benzene: —, fresh solution of neomethylbixin C; — — —, mixture of stereoisomers after insolation for 15 min. (From J. Am. Chem. Soc. **66**, 327 (1944))

second *trans* $\rightarrow$ *cis* rotation and gives neo C rather than yield marked amounts of the all-*trans* compound. On the other hand, all-*trans*-methylbixin is easily converted into neo A and *vice versa*. These isomerizations obviously affect another double bond than that which is *cis* in natural methylbixin. Therefore, we have the following scheme, in which the dotted arrows indicate hindered

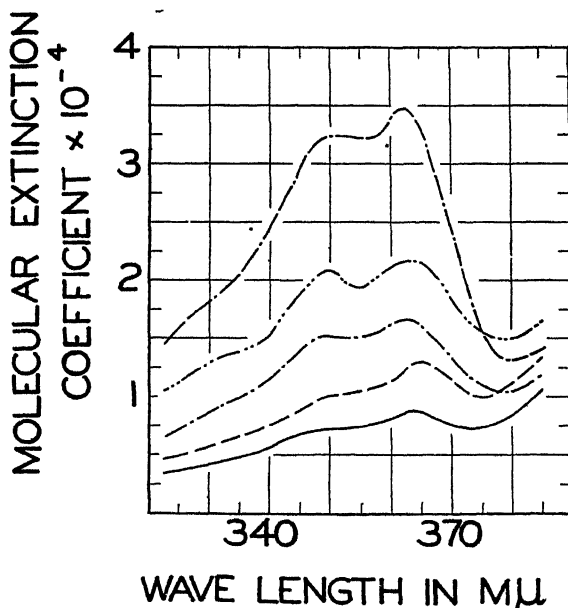
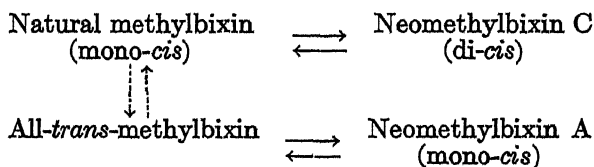


FIG. 37. Molecular extinction curves of some members of the stereoisomeric methylbixin set in the *cis*-peak region in benzene: —, all-*trans*-methylbixin; ---, natural methylbixin; —·—, iodine equilibrium mixture; ···, neomethylbixin C; ———, neomethylbixin A. (From J. Am. Chem. Soc. 66, 327 (1944))

interconversion and the full arrows easy interconversions, if the pigment is refluxed or insolated.



The molecular extinction coefficients $\times 10^{-4}$ at the *cis*-peak wave length (figure 37), minus the corresponding value for the all-*trans* compound, are as follows:

Natural methylbixin.	0.4	Neomethylbixin A.	2.8
All- <i>trans</i> -methylbixin.	0	Neomethylbixin C.	1.4

For neo B, because of its lability, it can be only claimed qualitatively that its extinction curve is flat in the *cis*-peak region.

On the basis of the spectral data, the mono-*cis* compound A, because of its exceptionally high *cis*-peak, must be assigned the configuration of 5-mono-*cis*-methylbixin. Natural methylbixin, which possesses an increased adsorption affinity and a low *cis*-peak, must have (in analogy to neo- β -carotene U) a peripherally located *cis* double bond and is 2-mono-*cis*-methylbixin. On the basis

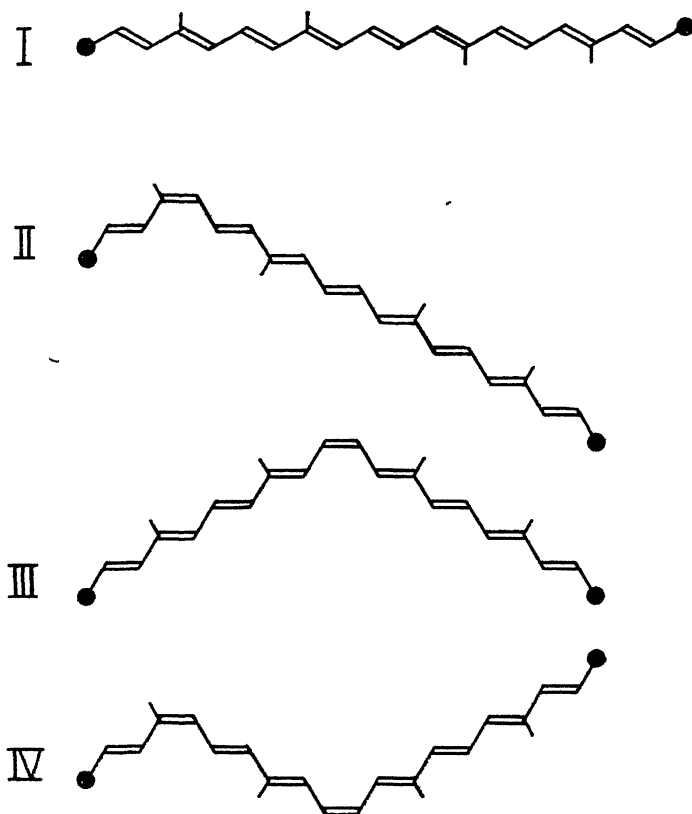


FIG. 38. Suggested stereochemical structures of the four crystalline members of the methylbixin set: I, all-*trans*-methylbixin; II, natural methylbixin; III, neomethylbixin A; IV, neomethylbixin C. The carboxyl groups are represented by black circles. (From J. Am. Chem. Soc. 66, 327 (1944))

of the scheme given on p. 331, it can be claimed that neo C is probably 2,5-di-*cis*-methylbixin. The skeleton models of these isomers are given in figure 38.

The above assignment of configurations is in reasonable agreement with the ratio of the observed principal absorption peaks in the visible region. For the four main members of the methylbixin set the experimentally found maximum intensity ratios are all-*trans*: natural: neo A:neo C = 1:0.93:0.80:0.82, while the ratios calculated according to Pauling (62) on the basis of the models (with the single-bond-double-bond angles taken as $125^{\circ} 16'$) are 1:0.87:0.82:0.79.

This agreement is satisfactory, considering the experimental error and the approximate nature of the theory.

Our configurational assignments (111) are also in accordance with some earlier data of the literature. Already van Hasselt (13, 14) has shown that the molecule of naturally occurring bixin possesses two non-equivalent terminal groups because it forms two different methyl ethyl esters. This feature was first explained by a structurally non-symmetrical bixin formula. Karrer, Helfenstein, Widmer, and van Itallie (21), however, correctly pointed out that the cause is stereochemical. Consequently, the *cis* double bond cannot have a central location in natural methylbixin and one of three possible positions appears to be excluded by a purely chemical argument.

B. CROCETIN

The first observation of stereoisomeric crocetins was made by Kuhn and Winterstein (39, 40) in 1933. They isolated dimethylcrocetin by extraction and exchange esterification of the natural crocetin gentiobioside (crocine) with methyl alcohol and obtained a second dimethylcrocetin with much lower melting point (141°C.), in addition to the well-known compound (m.p. 220°C.). The yield of the new isomer was 1 g. from 1 kg. of dry saffron, while the ordinary dimethylcrocetin can be isolated in quantities of 17 to 60 g. This result shows that two stereoisomeric crocetins must be present in the stigma. Unfortunately, it came to light later (32, 33) that the specimen of saffron used in the experiments mentioned was rather exceptional, and that most samples contain only the higher-melting isomer.

The biologically important occurrence of both *cis*- and *trans*-dimethylcrocetins in some algae, where they are active as sex-stimulating and directing agents, can only be mentioned; some fundamental observations were made by Kuhn, Moewus, *et al.* (27, 32, 33, 34, 35).

Because there is no generally accepted nomenclature in this field, the following terms have been used:

(a) Ordinary crocetin = *trans*-crocetin = stable crocetin = crocetin I

(b) *Cis*-crocetin = labile crocetin = crocetin II (known in form of derivatives)

The monomethyl and dimethyl esters of (a) were earlier termed β -crocetin and γ -crocetin, respectively, while the free dicarboxylic acid itself was called α -crocetin. This nomenclature is now obsolete.

The isomer (a) is certainly the all-*trans* compound; nothing seems to be known about the position or even the number of *cis* double bonds in (b). The spectral differences indicate one such bond (table 23, p. 334).

According to Kuhn and Winterstein (39), the labile dimethylcrocetin has a great tendency to rearrange and thus to form the stable isomer. This conversion can be achieved by the following methods: If the *cis* pigment is heated above its melting point (141°C.) and cooled, crystals of the *trans* form appear. These crystals possess a higher melting point (220–1°C.) than the starting material.

If a concentrated petroleum ether solution of the *cis* form is heated in the presence of a trace of iodine, crystals of *trans*-dimethylcrocetin are deposited within a short time.

The same *cis* $\rightarrow$ *trans* isomerization can be achieved very easily also by irradiation. Indeed, even during measurements in the visual spectroscope, the bands migrate toward longer wave lengths until they reach approximately those positions characteristic of the stable form. The spectral region between 400 and 500 $m\mu$ is the most effective, while ultraviolet or red light does not cause stereoisomerization. Irradiation of a petroleum ether solution with a Nitra-Osram lamp (9 volts, 3.9 amperes) at a distance of 35 cm., shifted the first spectral band from 446 $m\mu$ to 450.5 $m\mu$ within 15 min., by which time the process had been completed. The photosensitivity in a number of other solvents is similar.

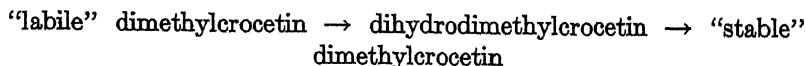
If labile dimethylcrocetin is boiled with zinc dust in pyridine in the presence of some glacial acetic acid, the color changes from orange-yellow to sulfur-yellow. Upon the addition of sodium hydroxide, the liquid becomes dark greenish blue,

TABLE 23
Comparison of "stable" (all-*trans*) and "labile" (mono-*cis*) dimethylcrocetins (39)

PHYSICAL PROPERTIES	STABLE FORM		LABILE FORM	
Crystal form.....	Hexagonal plates		Long, rectangular	
Color under the microscope.....	Reddish orange		Yellow	
Melting point (corrected), °C.....	222		141	
Solubility in methanol at 20°C.....	1:100,000		1:6,000	
Solubility in ether.....	Lower		Higher	
Spectral maxima in petroleum ether (b.p. 70–80°C.).....	450.5	424.5 $m\mu$	445	422 $m\mu$
Spectral maxima in chloroform.....	463	434.5 $m\mu$	458	432.5 $m\mu$

and shaking in air instantaneously produces the orange color of the stable form.

Thus the following chemical transformation which is analogous to that in the bixin set, has been achieved:



We are able to predict that the reported stereoisomeric changes will be found to be reversible, and furthermore that several new members of the set can be observed and isolated. Chromatographic separation should offer no difficulty, and has indeed been claimed for the two dimethylcrocetins by Winterstein and Stein (99) as well as by Winterstein (98) but, unfortunately, experimental directions are lacking.

C. AZAFRIN

As pointed out by Kuhn and Winterstein (39), azafrin, $(\text{HO})_2\text{C}_{25}\text{H}_{35}\text{COOH}$, which occurs in the Azafranillo root (*Escobedia scabrifolia*, *E. linearis*, Schl.)

(28, 42) very probably possesses an all-*trans* configuration. Its methyl ester was regenerated unchanged in melting point and spectrum from an iodine addition product; the spectral bands of the natural pigment did not shift their position when illuminated.

IV. DIPHENYLPOLYENES

A. GENERAL REMARKS

In the series of the diphenylpolyenes, $\text{C}_6\text{H}_5(\text{CH}=\text{CH})_n\text{C}_6\text{H}_5$, some stereochemical aspects of the simplest representatives, stilbene ($n = 1$) and diphenylbutadiene ($n = 2$), were the subjects of early investigations, but until recently the data available referred only to these compounds.

The methods of synthesis for higher members, up to $n = 8$, were, to a large extent, developed by Kuhn and Winterstein (37; for $n = 15$ see 36a). They found that even when a higher diphenylpolyene was synthesized by ten different methods, the same compound was isolated in each instance. Evidently the conditions favor the formation of the most stable stereoisomer in each set, i.e., the "ordinary" diphenylpolyene. That this is the all-*trans* member of the set has been proved for diphenylhexatriene and diphenyloctatetraene by the x-ray studies of Hengstenberg and Kuhn (15).

According to Kuhn and Winterstein (37), in this unbranched and symmetrical series the number of possible stereoisomers, N , of a compound which contains n double bonds is

$$N = 2^{n-1} + 2^{p-1}$$

where $n = 2p$ for an even number and $n = 2p - 1$ for an odd number of aliphatic conjugated double bonds.

It has, however, recently been pointed out by LeRosen and the author (114, 115) that by no means all stereoisomers may be formed with equal probability. All possible members of a given stereoisomeric set can be classified into two types: one type (*a*) possesses *cis* configuration about one or both double bonds adjacent to the benzene rings, while the other (*b*) shows *trans* configuration in these bonds.

The probability of the formation in any substantial quantity of isomers belonging to type (*a*) is much smaller because of the steric conflict represented in figure 39. This figure shows that a hydrogen atom of the ring (in ortho position to the side chain) is spatially hindered by one of the side-chain hydrogens, so that an approximately planar configuration becomes impossible. The deviation will be about 52.5° . Therefore, if an all-*trans* diphenylpolyene isomerizes, the formation of those members which possess *trans* configuration on both ends of the aliphatic system will be favored.

In the limiting case of *cis*-stilbene (47), hydrogen atoms attached to different rings interfere with each other (figure 40). The difference in optical properties of *cis*- and *trans*-stilbenes has been interpreted by Lewis and Calvin (46) on the assumption that the repulsion of the two ortho hydrogen atoms forces the mole-

cule out of a plane and diminishes resonance. On a similar basis the energy difference between the two stereoisomers (10 kcal.) can be explained (Lewis, Magel, and Lipkin (47)).

In the stilbene and diphenylbutadiene sets only sterically hindered stereoisomers are possible; in higher diphenylpolyenes, however, the *trans* $\rightarrow$ *cis* isomerization of the all-*trans* form will be distributed between both types. The isomers which can be formed without significant steric hindrance will, however, be preferred and their formation can therefore be expected in substantially larger quantities than those which involve a definite spatial conflict. This

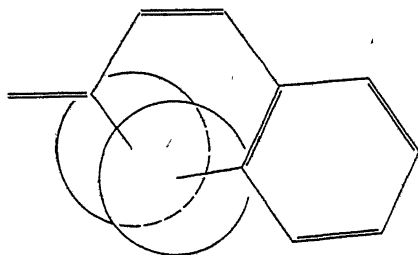


FIG. 39. Model of one molecule end of diphenyloctatetraene. Values used: $\text{C}=\text{C}$, 1.33 Å.; $\text{C}-\text{C}$, 1.46 Å.; $\text{C}_6\text{H}_5-\text{C}$, 1.44 Å.; $\text{C}_6\text{H}_5-\text{H}$, 1.08 Å.; $\text{C}-\text{H}$, 1.09 Å.; H radius, 1.20 Å.; angles $\text{C}=\text{C}-\text{C}$ and $\text{C}-\text{C}-\text{H}$, $124^\circ 20'$. (From J. Am. Chem. Soc. 64, 2757 (1942))

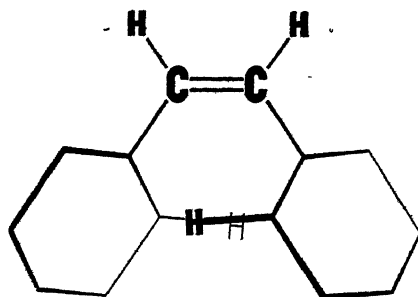


FIG. 40. Overlapping of hydrogen atoms in *cis*-stilbene (From J. Am. Chem. Soc. 62, 2977 (1940))

prediction is in accordance with some observations in the diphenyloctatetraene set to be discussed below (p. 339).

The number of possible stereoisomers of each type is given for certain diphenylpolyenes in table 24.

B. 1,2-DIPHENYLETHYLENE (STILBENE AND ISOSTILBENE), $\text{C}_6\text{H}_5\text{CH}=\text{CHC}_6\text{H}_5$

As is well known, the literature dealing with this simplest polyene is extensive, but the discussion of the following data may suffice.

Cis-stilbene, also termed "isostilbene", which was detected by Otto and

Stoffel (59) as early as in 1897, may be prepared either by photochemical or by purely chemical methods. Stoermer (79) obtained a small quantity of isostilbene from the *trans* form by prolonged irradiation with ultraviolet light, and a similar method was used recently by Lewis, Magel, and Lipkin (47). The quantum yields of such an irradiation were studied by Smakula (74). Stoermer and Prigge (80) early reported data concerning the effect of irradiation on some stilbene derivatives.

The older chemical method for the preparation of isostilbene, *viz.*, the partial reduction of diphenylacetylene (tolane), has now gone into discard in favor of the Stoermer method employing decarboxylation of α -phenylcinnamic acid:

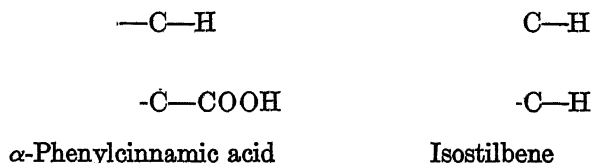


TABLE 24

The two types of *cis*-diphenylpolyenes, $\text{C}_6\text{H}_5(\text{CH}=\text{CH})_n\text{C}_6\text{H}_5$

STEREoisomeric SET	n	NUMBER OF STEREOISOMERS		
		Hindered	Not hindered	Total
Stilbene.....	1	1	0	1
Diphenylbutadiene.....	2	2	0	2
Diphenylhexatriene.....	3	4	1	5
Diphenyloctatetraene.....	4	7	2	9
Diphenyldecapentaene.....	5	14	5	19
Diphenyldodecahexaene.....	6	26	9	35

By the use of a copper chromite catalyst, Taylor and Crawford (89) have succeeded in improving this conversion so effectively that pure *cis*-stilbene can now be prepared in quantity, since the yields amount to 60–65 per cent.

The preparation of ring-substituted *cis*-stilbenes follows the same lines. For example, Ruggli and Staub (70) obtained nitro and amino derivatives; Späth and Kromp (77) prepared *cis*-pterostilbene by decarboxylation.

Although *cis*-stilbene had been described as an oil, Weygand and Rettberg (94) recently succeeded in obtaining a crystalline sample (m.p. 1°C.) by fractional precipitation with 1,3,5-trinitrobenzene, after a systematic elimination of such contaminants as tolane, dibenzyl, and *trans*-stilbene. Pure *cis*-stilbene is more thermostable than might be expected. Even at 214°C. only 8 per cent undergoes isomerization in 20 hr. (Taylor and Murray (90)). The kinetics of this process has been studied by Kistiakowsky and Smith (25).

In alcoholic solution *cis*-stilbene has its spectral maximum at 278 $m\mu$ and a molar extinction coefficient of 0.935×10^4 . The maximum of the *trans* compound lies at a considerably longer wave length (294 $m\mu$) and the molar extinc-

tion is 2.34×10^4 . These data are comparable with those observed with the *cis*- and *trans*-cinnamic acids (5, 48, 75). For a new investigation in this field cf. Jones (19).

Cis-stilbene is easily isomerized by iodine (a process which is promoted by sunlight) and by other catalysts, for example, boron trifluoride (Price and Meister (68)). Kharasch, Mansfield, and Mayo (24) observed, however, that it is not isomerized by hydrobromic acid in the dark, either in the presence or in the absence of air. In contrast, the addition of benzoyl peroxide, ascaridole, or other peroxidic compounds to this system caused a rapid *cis* $\rightarrow$ *trans* change. Although *cis*-stilbene solutions are isomerized by hydrobromic acid in light, the process is less rapid in the absence of air. Because antioxidants, e.g., hydroquinone, hinder this *cis*-*trans* rotation, it is assumed that bromine atoms, liberated from the acid by light, oxygen, or a peroxide, are responsible for the process. The ineffectiveness of hydrochloric acid may be explained by its relatively high stability toward oxygen. A thorough discussion of related phenomena has appeared in this journal (Mayo and Walling (51); see also 90a).

For the practical separation of *cis*-stilbene and *trans*-stilbene use has been made of fractional distillation and crystallization, and recently of molecular addition products (Weygand and Siebenmark (95)). Spectrophotometric measurements, melting-point curves, and other methods (25, 47, 60, 68, 90, 94, 95, 95a) may be employed for the determination of the *cis* and *trans* components in mixtures. For the formation of mixed crystals see reference 93a.

It was reported recently by McNeely and the author (118) that a convenient procedure for the detection, purification, separation, and estimation of stereoisomeric stilbenes is to be found in the so-called chromatographic brush method (108, 112). After extrusion of the Tswett column containing an invisible chromatogram, a narrow streak is made down the cylinder with a brush which has been dipped into a 1 per cent permanganate solution. Where the reagent crosses a zone containing *cis* or *trans*-stilbene, it turns brown almost instantaneously. The zones thus located can be cut out and the isomers eluted after the streak has been shaved off. The application of this method to stilbene, *p*-methylstilbene, and *p*-methoxystilbene showed that in each case the *trans* form possesses the stronger adsorption affinity and is located near the top of the alumina column. Following this procedure, samples of *cis*- or *trans*-stilbene can be chromatographically tested for possible contamination by the other isomer. In a small-scale experiment the limit of detection is 1 to 2 per cent.

C. 1,4-DIPHENYLBUTADIENE, $C_6H_5(CH=CH)_2C_6H_5$

The three expected steric forms of this compound, *trans-trans*, *trans-cis*, and *cis-cis*, have been obtained by Straus (87) in the following way: When diphenyldiacetylene, $C_6H_5C\equiv C-C\equiv CC_6H_5$, is boiled with copper-coated zinc dust, a labile isomer of diphenylbutadiene is formed, which on long standing (or more rapidly in sunlight) can be converted into ordinary all-*trans*-diphenylbutadiene. In order to prepare the third member of the set, the reduction of diphenyldiacetylene was interrupted prematurely. The dihydro derivative

formed, $C_6H_5C\equiv C-CH=CHC_6H_5$, was further reduced and yielded a new labile diphenylbutadiene distinctly different from that just mentioned. Since this oily isomer is the most labile of the set and is easily converted by insolation into the crystalline all-*trans* form within 15 min., it may be reasonably assumed that it is the *cis-cis* form. The *trans-cis* configuration should be assigned to the isomer obtained by the copper-zinc method.

D. 1,6-DIPHENYLHEXATRIENE, $C_6H_5(CH=CH)_3C_6H_5$

No record of successful stereoisomerization of the triene seems to be available. However, the methods described below for the tetraene will very probably be satisfactory.

E. 1,8-DIPHENYLOCTATETRAENE, $C_6H_5(CH=CH)_4C_6H_5$

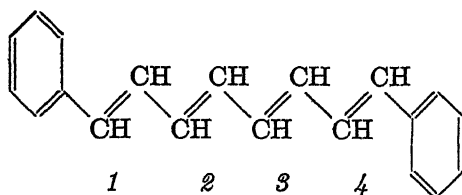
Until recently only the ordinary, all-*trans* form of this polyene was known. An alleged "white modification", observed earlier (78) and occasionally still mentioned in the literature (7), has been identified by Kuhn and Winterstein (37) as stilbene.

The stereochemical homogeneity of tetraene samples prepared by the customary methods of synthesis (37) can be demonstrated chromatographically; a single, fluorescing zone appears on the lime column. It was found in collaboration with LeRosen (114, 115), that it is easy to stereoisomerize all-*trans*-diphenyloctatetraene by some methods described in Part II. The mixture of stereoisomers obtained can be separated chromatographically on calcium hydroxide. After a few hours' refluxing in benzene and after development with petroleum ether containing 10 per cent of benzene, three fluorescing zones became visible when the column was illuminated with ultraviolet light. The weight ratio of the isomers, from top to bottom, was 83:15:2, i.e., about one-sixth of the starting material had undergone a spatial change. The top zone contains unchanged all-*trans* compound, while in the two other zones the two "unhindered" members of the set seem to be present (*cf.* table 24). It is reasonable to assume the following configurations (from top to bottom) (see the formula on p. 340):

<i>trans-trans-trans-trans</i>	= all- <i>trans</i> -diphenyloctatetraene
<i>trans-cis-trans-trans</i>	= 2- <i>cis</i> -diphenyloctatetraene
<i>trans-cis-cis-trans</i>	= 2,3-di- <i>cis</i> -diphenyloctatetraene

In favorable cases two minor zones appeared below these main stereoisomers and very probably contained some of the spatially "hindered" members of the set.

Similar chromatograms can be obtained by treating the benzene solution of diphenyloctatetraene with some iodine for 15 min. or by keeping the melted crystals at 260°C. for the same time. Irradiation of solutions for 12 hr. with a mercury quartz lamp yielded, in addition to the main isomers, two minor members with markedly weaker adsorption affinity.

All-*trans*-diphenyloctatetraene

(The sterically effective double bonds are numbered; 1 and 4 are of the hindered type)

The bending of the tetraene molecule can also be demonstrated by means of the *cis*-peak effect. Upon addition of iodine to the benzene solution of the all

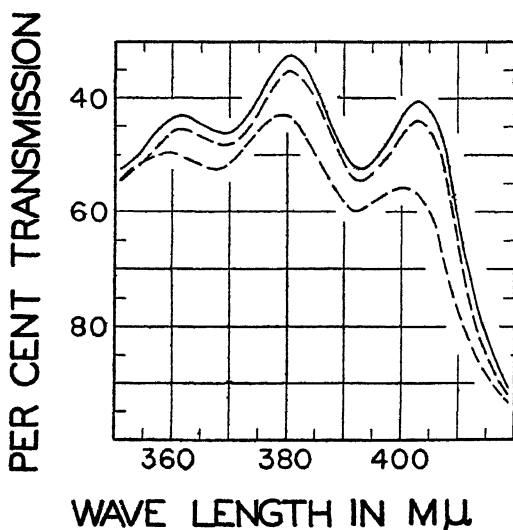


FIG. 41. Spontaneous re-isomerization of 2-*cis*-diphenyloctatetraene (and minor isomers) in benzene at room temperature in darkness. The transmission curves from bottom to top were taken after 0, 1, and 2 days, respectively. The upper curve remained constant on the fourth day.

trans compound, the peak appears at 286–288 mμ. Illumination, preferably with an ultraviolet lamp, is needed for this process.

All diphenyloctatetraenes possessing *cis* double bonds thus far observed are labile (115), and it is therefore understandable that they have never appeared in the course of syntheses. Even with a knowledge of their properties, it has been impossible up to the present to prepare crystalline samples for which the absence of some all-*trans* form could be guaranteed. Chromatographic analysis showed that fresh eluates of the partially-*cis* zones mentioned contained only traces of the all-*trans* isomer which, however, rapidly increased in quantity on standing, even at 5°C., in darkness.

The spontaneous re-isomerization of 2-*cis*-diphenyloctatetraene is demon-

strated in figure 41, which shows as a function of time the gradual increase in the light extinction at room temperature.

Such a re-isomerization is especially rapid in ultraviolet light. If a Tswett column containing adsorbed *2-cis* form is irradiated for a few minutes with an ultraviolet lamp, further development splits it into two fluorescing zones. The inside of the corresponding section of the column, however, does not contain all-*trans*-tetraene, which was formed only locally by irradiation of the surface.

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¹ Owing to present conditions, this bibliography is not claimed to be exhaustive.

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CATALYSIS FROM THE VIEWPOINT OF THE ELECTRONIC THEORY OF ACIDS AND BASES¹

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I. INTRODUCTION

One of the primary questions that theoretical chemistry has always concerned itself with is: How do reactions take place? The answer to this question has been sought with renewed vigor during the last two decades. Stimulated by the Lewis theory of the covalent bond, chemists have gone far toward answering the question for many reactions.

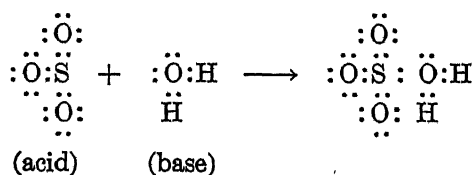
¹ Presented as part of the Symposium on Molecular Addition Compounds before the Division of Physical and Inorganic Chemistry at the 106th Meeting of the American Chemical Society, Pittsburgh, Pennsylvania, September 6, 1943.

The point now seems to be reached, however, at which the lack of a comprehensive understanding of the terms "acid" and "base" may be a handicap to further progress. An adequate terminology is essential to the progress of any science. Not only is such a terminology necessary to deal with knowledge as it is gained, but it often suggests new interrelationships with other branches of the science which may in turn lead to new discoveries.

Fortunately the basis of this terminology has already been provided by the electronic theory of acids and bases (43, 44, 51), which although proposed twenty years ago has been largely ignored until recently (45-49, 51). This theory systematizes a widespread range of chemical phenomena. It fits a large number of apparently isolated data into a more complete and clear picture which, like a jig-saw puzzle, appears simple once the scattered pieces have been fitted together.

The one-element theory in various forms has been a persistent idea throughout the history of chemistry. Some of us can remember when the chemical behavior which we call oxidation was supposed to depend upon one element. We no longer hold to the one-element theory of oxidation-reduction, but we all know how difficult it is to give up the one-element theory of acids and bases. Of course at one time that element was oxygen, but it seems that when chemists changed from oxygen to hydrogen they decided never to change again. However, the experimental facts are compelling us to realize that the presence of a particular element in a substance is not essential for acid properties.

The electronic theory of acids and bases follows from Lewis' definitions based on these experimental facts (43, 44, 51). According to this theory a substance is acting as an acid when it accepts a share in a lone electron-pair from a base to form a coordinate bond. Any substance capable of donating a share in a pair of electrons may act as a base toward a sufficiently strong acid. The formation of the coordinate bond is neutralization. One example will be given here; others have been cited previously (44, 51).



Subsequent ionization depends upon several factors, including the dielectric constant and solvating power of the solvent—if a solvent is present.

We conclude that almost any substance may behave as an acid or a base. The majority of reagents, under properly chosen conditions, may react in either way, i.e., are amphoteric. There are a few exceptions such as the "inert" gases, which so far as we know can act only as extremely weak bases (10). But as a rule, most substances can be made to act as bases if reacted with a sufficiently strong acid (stannic chloride or sulfur trioxide will compel even hydrogen chloride to act as a base (51)), and many substances can be made to act as acids if reacted with a sufficiently strong base. Perhaps we should give up the terms "acid" and "base" and instead use "acidity" and "basicity" as referring to a particular

reaction. But probably the convenience of using the nouns makes their retention worth while if we are careful to remember the importance of two other nouns—relativity and amphoterism.

The use of the terms "electrophilic" and "nucleophilic" is already well known. By changing nucleophilic to electrodotic (52) and using the Lewis theory of acids and bases we have the basis of a new terminology which includes both oxidation-reduction and acid-base phenomena as manifestations of the electrophilic and electrodotic properties of substances in general (52). Depending upon the conditions of the particular reaction, a large number of substances may act either as oxidizing agents or as acids (electrophilic reagents). Another large group of substances may act either as reducing agents or as bases (electrodotic reagents). Many in each group may react in all four ways. Water acts as an acid toward ammonia, as an oxidizing agent toward sodium, as a base toward sulfur trioxide, and as a reducing agent toward fluorine (table 1).

TABLE 1
Examples of electrophilic and electrodotic reactions of water

Electrophilic
$\text{H}_2\text{O} + \text{NH}_3 \longrightarrow \text{NH}_4^{+1} + \text{OH}^{-1}$ (acid)
$2\text{H}_2\text{O} + 2\text{Na} \longrightarrow 2\text{Na}^{+1} + 2\text{OH}^{-1} + \text{H}_2$ (oxidant)
Electrodotic
$\text{H}_2\text{O} + \text{SO}_3 \longrightarrow \text{H}^{+1} + \text{HSO}_4^{-1}$ (base)
$2\text{H}_2\text{O} + 2\text{F}_2 \longrightarrow 4\text{H}^{+1} + 4\text{F}^{-1} + \text{O}_2$ (reductant)

Many examples showing how the Lewis theory conforms to the experimental data have already been given (44, 51), but catalysis is one aspect of the subject which has not been dealt with systematically. This paper represents an attempt to do that. Familiar examples of acid-base catalysis are discussed from the Lewis viewpoint in order to show that there is no essential difference in catalytic behavior whether or not protons are involved.

"Catalysis" as usually defined has implied that the catalyst is recovered unchanged. Recently, there seems to be a tendency to broaden the definition. For example, Hammett (25) points out that there is no important distinction between catalysis in the older sense and the kind of acceleration produced by a base which appears as its "conjugate acid" at the end of the reaction. A few of our examples are of the latter type, but most of them conform to the older definition.

Since it is already generally recognized that basic behavior is displayed by a very large number of substances, only enough examples of base-catalyzed reac-

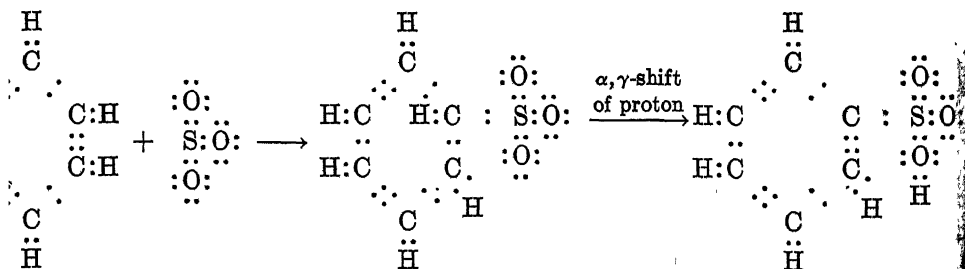
tions are included in this paper to show that when a base acts as a catalyst the substance attacked by the base is behaving as an acid.

Many examples of typically acidic properties displayed by substances which do not contain protons have already been listed (44, 51), but the idea that acidic behavior does not depend upon protons and, in fact, is as widespread as basic behavior is not yet widely accepted. So, although a few more such examples are mentioned in this paper, the principal emphasis is upon systematic application of the new theory to acid catalysis.

II. ACID CATALYSIS

Evidence has been cited previously to show that the common Friedel-Crafts type catalysts such as aluminum chloride, ferric chloride, stannic chloride, and zinc chloride are acids (51). Their catalytic activity is well known in connection with organic reactions, but it is not usually recognized that they also catalyze such common reactions as those of metals with water. The reaction of iron with pure water to give hydrogen is a very slow one because the concentration of hydrogen ions (hydrated, of course) is so low. Any acid sufficiently strong to increase the hydrogen-ion concentration substantially will accelerate the reaction. It is immaterial whether the acid is molecular hydrogen chloride, acetic acid, sulfur trioxide, carbon dioxide, stannic chloride or ionic zinc chloride or cupric sulfate. The rate of reaction depends upon increasing the hydrogen-ion concentration, which in turn depends upon the concentration and strength of the acid which is displacing protons from the water. We shall see that this increase in positive-ion concentration is typical of acid catalysis. Obviously these ideas may have a far-reaching effect on the study of corrosion. Corrosion of metals is a complex subject, but it would seem that application of the electronic theory of acids and bases might be helpful in its investigation.

The Friedel-Crafts catalysts are usually thought of as halides of a few metals, so a natural question to raise might be: If these halides are really acids differing in no fundamental manner from H-acids in their behavior, why do not H-acids catalyze reactions of the Friedel-Crafts type also? The answer is that they do. Hydrogen fluoride (78), phosphoric acid, and sulfuric acid (14, 32) have been established as catalysts for Friedel-Crafts and similar reactions.



The catalytic activity of sulfuric acid seems especially interesting. Depending on the concentration of water or of sulfur trioxide, either sulfur trioxide or the

H-acid or both may be responsible for the catalysis. Lewis and Bigeleisen² have shown (10) that the very rapid increase in the acidity function of Hammett and Deyrup as 100 per cent sulfuric acid is approached is probably due to sulfur trioxide. This might indicate that in the sulfonation of benzene the sulfur trioxide is the principal agent in a direct electrophilic attack.

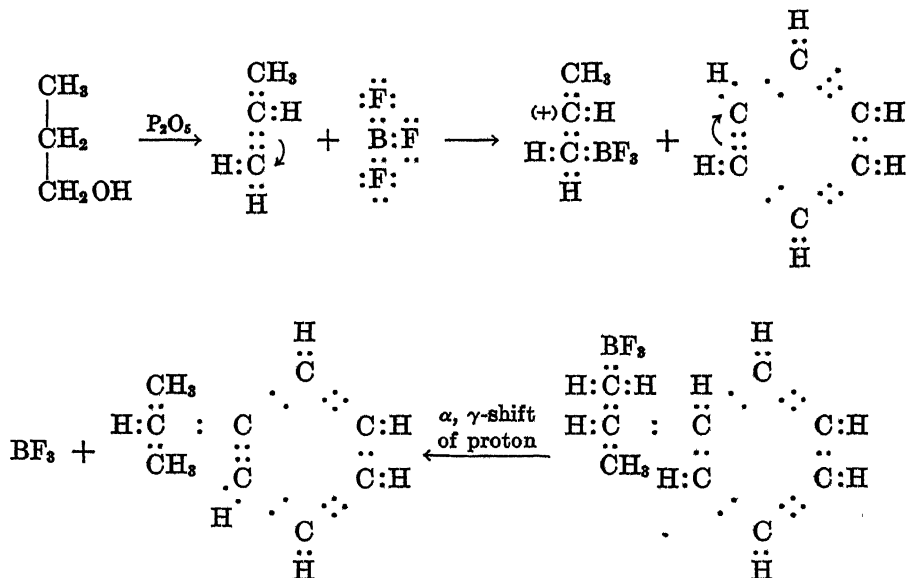
The examples listed below have been chosen because of their familiarity to most chemists. The application of a new terminology to well-known facts is always helpful in acquiring facility in its use.

A. FRIEDEL-CRAFTS REACTIONS

The mechanisms of Friedel-Crafts reactions have been studied extensively. The alkylations involve the use of olefins, alkyl halides, alcohols, ethers, and esters. The acylations make use of acids, esters, acid halides, and acid anhydrides. This type of reaction is *acid-catalyzed*, using such compounds as boron, aluminum, iron, tin, and other metallic halides, as well as sulfuric acid, phosphorus pentoxide, orthophosphoric acid, and hydrogen fluoride. These act as acids in the catalytic activity described because they all have a strong tendency to accept a share in an electron-pair as the first step in the reaction.

1. Alkylations

(a) *Alcohols*: Toussaint and Hennion (91) alkylated benzene with alcohols, using boron trifluoride as the acid catalyst and phosphorus pentoxide, sulfuric acid, and benzenesulfonic acid as assistants. They explained the mechanism on the basis of a dehydration of the alcohol to an olefin:

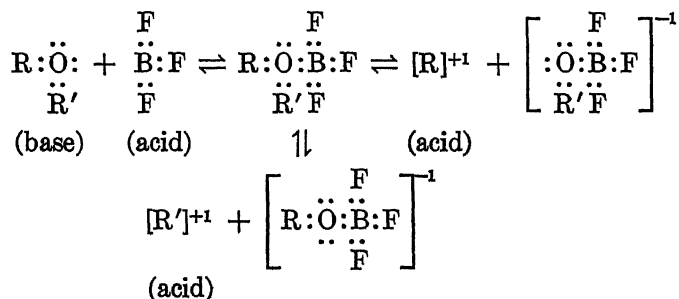


² The similarity of the absorption curves for methylene blue in stannic chloride and in sulfuric acid, given by the same authors, is a striking demonstration of the essential likeness in the behavior of these two acids toward indicators.

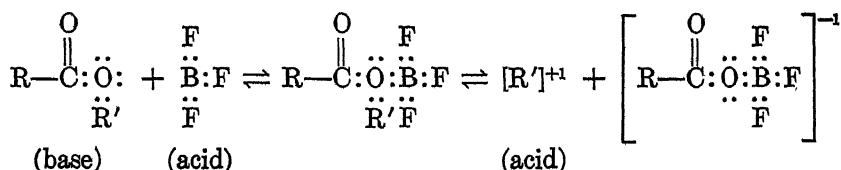
the similarity between boron trifluoride and H-acids. It is interesting to note that hydrogen fluoride does not effect methylations, although aluminum chloride does (84). This seems to indicate that aluminum chloride is a stronger acid than hydrogen fluoride.

(b) *Ethers*: The catalytic effect of boron trifluoride on alkylations with ethers (59, 63, 73) involves an acid-base reaction between boron trifluoride and ether. That this is a true acid-base reaction can be readily demonstrated by titrations in different solvents and with various indicators (54). The positive fragment that results from the ionization then reacts with the benzene, as previously described.

Hydrogen fluoride (80) has also been used to catalyze these reactions. Whether or not the acid contains hydrogen, the behavior is essentially the same. The acid accepts a share in a pair of electrons from the base to form a coördinate bond. Ionization into single ions may then occur if the dielectric constant is high enough and the ions can be solvated. In media of very low dielectric constant considerable association into higher ionic multiples is to be expected (22, 23, 53, 93), but this usually does not have to be indicated in the equations.



(c) *Esters*: The behavior of esters toward acids may result in an alkylation or an acylation depending on the relative tendencies of acyl, alkyl, and aryl groups to become acidic ions in the presence of aluminum chloride, boron trifluoride, metallic halides, or H-acids. The alkylation mechanism (11, 57, 62) can be represented as:

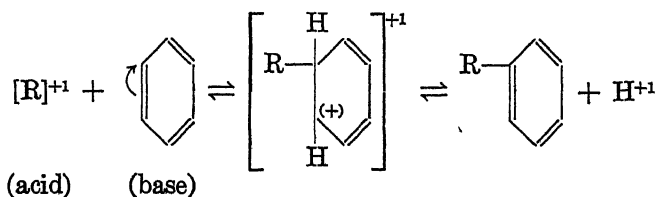


The acyl group, being more electrophilic than the alkyl (R') group, will not be released and, therefore, in the presence of an aromatic compound an alkylation will result.

However, if R' is an aryl group which is more strongly electrophilic, then the acyl group will be released as a carbonium ion and acylation will result. This reaction is discussed further under the section on acylation.

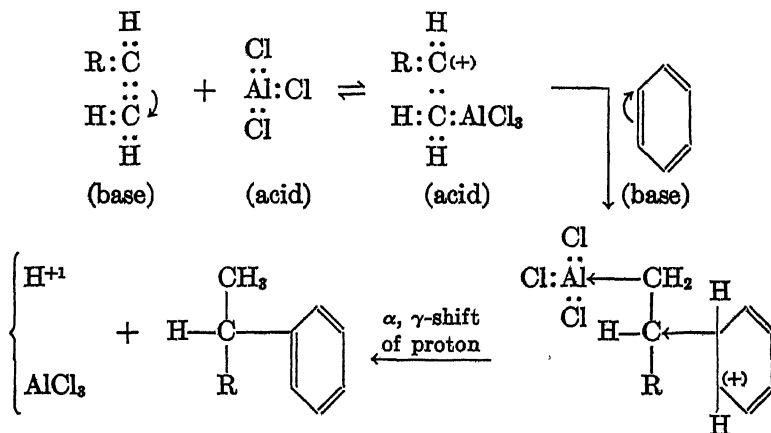
Burwell and Archer (14) report that although the alkyl chlorides and bromides are not catalyzed by boron trifluoride, the alkyl fluorides will react vigorously. They attribute this to the greater stability of the BF_4^- ion as contrasted with the BF_3Br^- ion or the BF_3Cl^- ion.

The "positive fragments" formed as a result of these acid-base reactions alkylate or acylate aromatic compounds through an electrophilic mechanism involving the electron-deficient carbonium ion (R^+) (69).



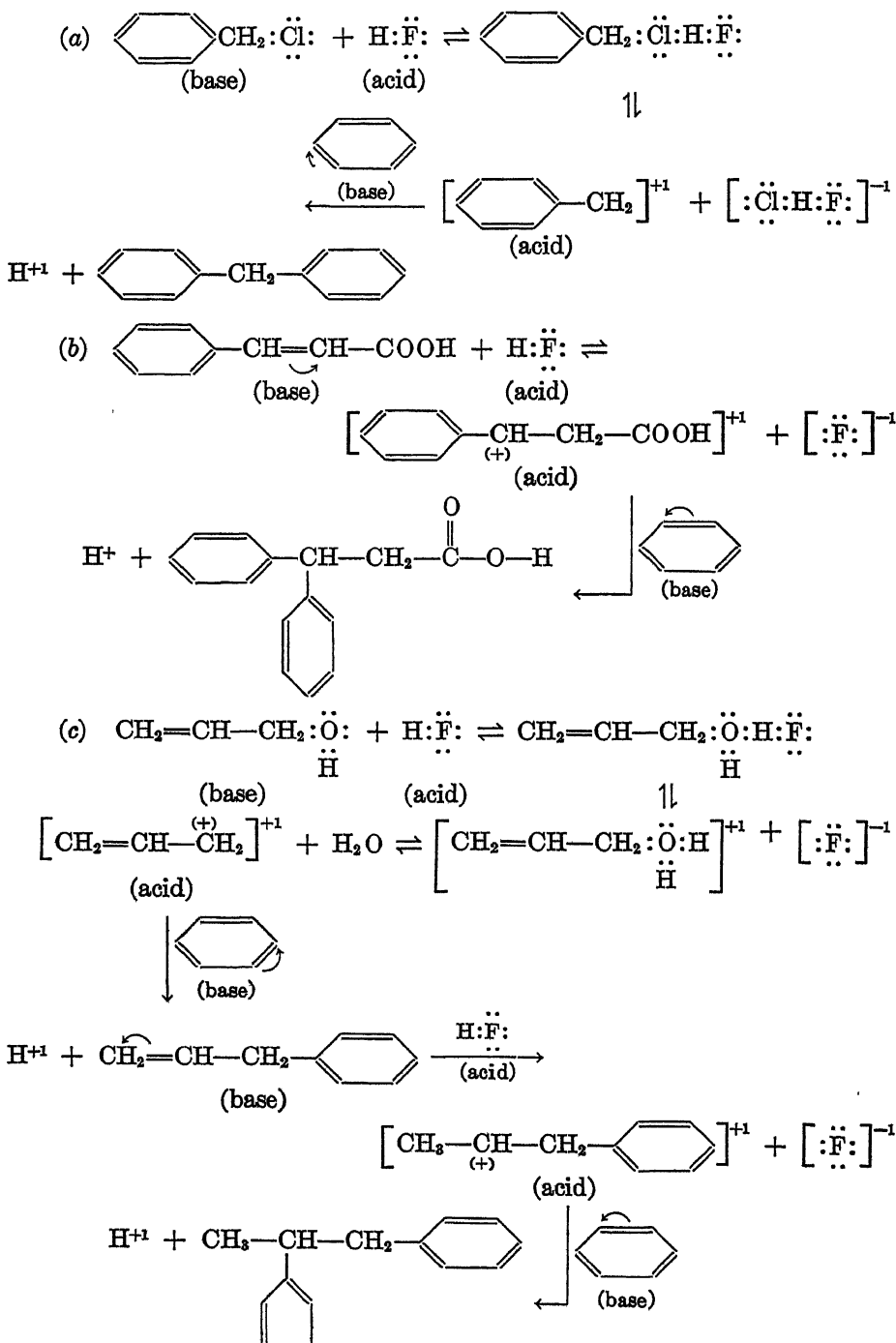
(e) *Olefins*: Olefins can be employed for the alkylations of aromatic compounds by using acid catalysts such as hydrogen fluoride (77), sulfuric acid (32), phosphoric acid (34), phosphorus pentoxide (92), boron trifluoride (33, 87, 103), and aluminum chloride (31, 72).

The condensation of olefins with aromatic compounds has been explained through an electrophilic mechanism involving the acid-base concept. The electrophilic (acidic) catalyst reacts with the electrodotic (basic) olefin to form an acidic intermediate which can react with the aromatic compound which is electrodotic. The reactions seem to be (69):



(f) *Allylic groups*: Simons and Archer (81) investigated the alkylations of benzene using benzyl chloride, cinnamic acid, and allyl alcohol as the alkylating agents and hydrogen fluoride as the acid catalyst. The products they obtained are in agreement with those that might be predicted on the basis of the polarization or polarizability of the alkylating agents. Boron trifluoride, stannic chloride, and other acid catalysts also will catalyze these alkylations.

Possible mechanisms might be as follows:

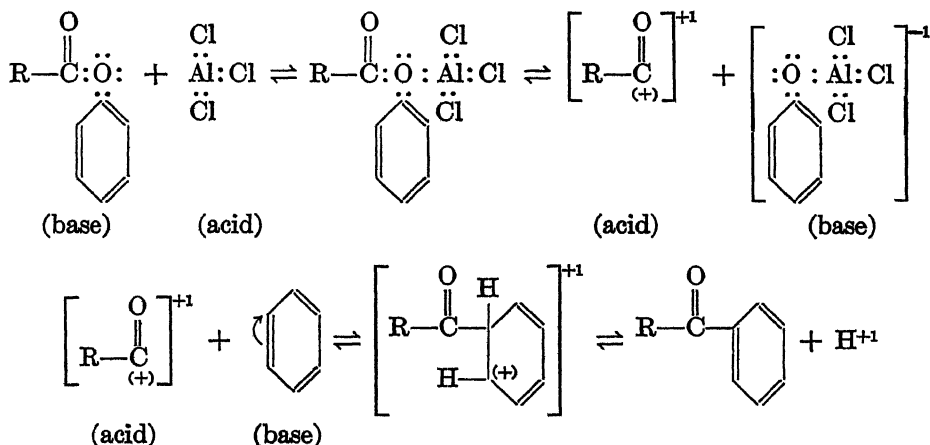


With allyl alcohol the first point of electrophilic attack is at the carbon-oxygen bond, thus producing an allyl carbonium ion which will attack a benzene molecule to produce the 3-phenyl-1-propene. This product is very susceptible to electrophilic attack by the catalyst because of the polarization of the molecule. Thus a carbonium ion would be produced that on reaction with benzene would produce the 1,2-diphenylpropane. The polarization indicated is supported by the fact that practically no 1,3-diphenylpropane is formed in the reaction.

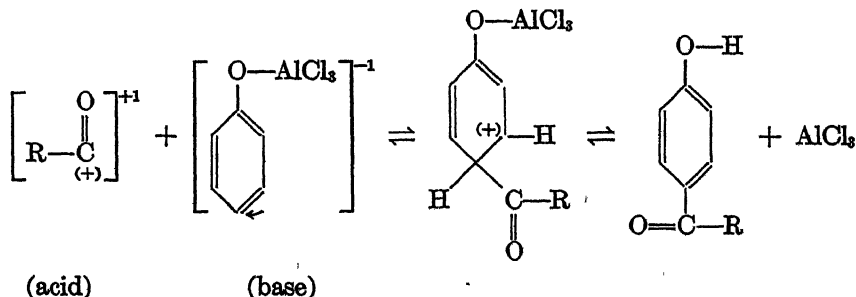
The use of sulfuric acid as an electrophilic catalyst produces much larger amounts of the 1,2-diphenylpropane and less of the 3-phenyl-1-propene.

2. Acylations

(a) *Esters*: Acylation by esters (11, 13, 17, 36, 57, 98), mentioned in a preceding section, can be catalyzed by aluminum chloride, boron trifluoride, metallic halides, and H-acids as catalysts. The possible mechanism is represented by the following equations:



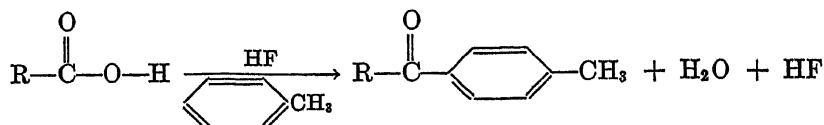
The Fries reaction (7) may be explained on the same basis, i.e.,



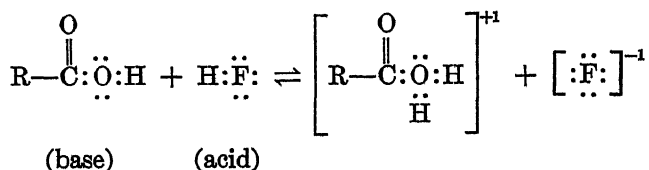
In this special case of an acylation there is an intramolecular reaction between the acidic acyl group and the electrodotic aromatic nucleus. The fact that ortho and para derivatives are obtained can readily be explained on the basis of

increased electron density at these positions, an increase which facilitates the electrophilic attack of the carbonium ion.

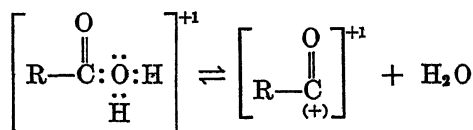
(b) *Acids*: Acids (86) have been employed for acylations using hydrogen fluoride as a catalyst:



Simons (76) reports that carboxylic acids dissolved in liquid hydrogen fluoride produce conducting solutions:

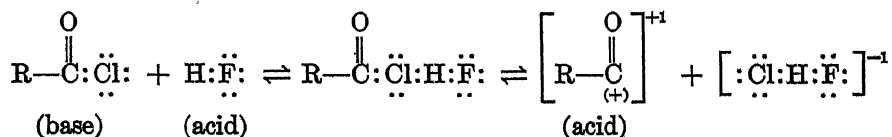


The resulting cation will then dissociate to form water and the acyl carbonium ion, which will acylate an aromatic compound through an electrophilic attack.

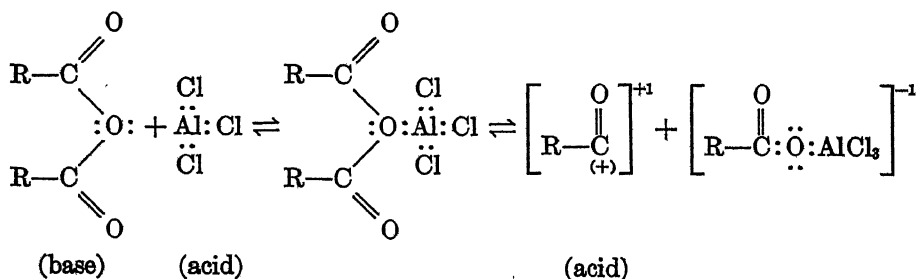


It will be noted that these weak carboxylic acids are being forced to act as bases by the much stronger acids used as catalysts.

(c) *Acyl halides*: The mechanism of the acylation reaction of the acid halides (76, 86) could also be explained on the basis of the following equation:

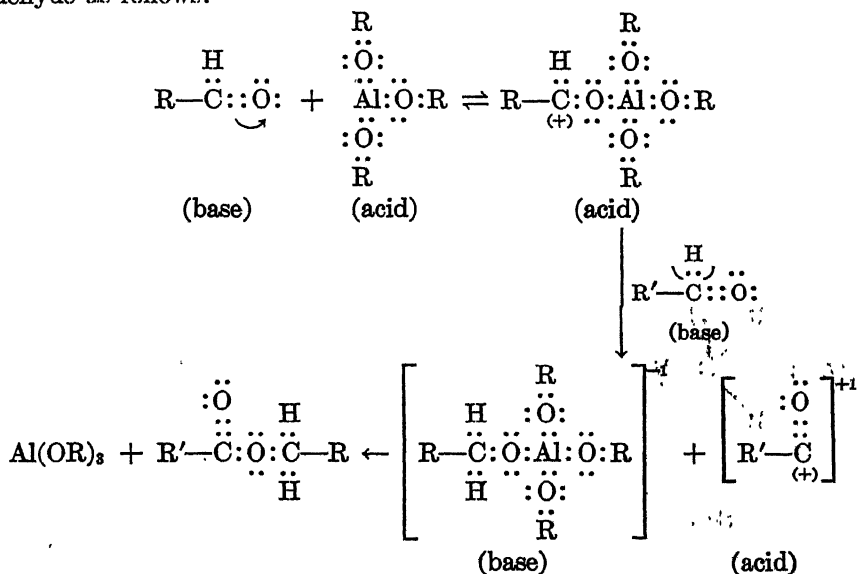


(d) *Acid anhydrides*: The mechanism of the acid-catalyzed acylations using an acid anhydride as the acylating agent could be written as follows:

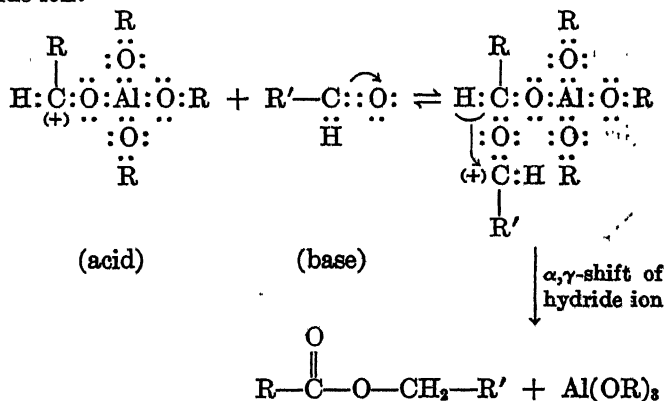


B. CANNIZZARO REACTION

A special case of the Cannizzaro reaction results in the direct formation of an ester from two molecules of an aldehyde (15, 90). The catalytic agent used here is a generalized acid. The aluminum ethylate coördinates with a molecule of the aldehyde, acting as a base, and produces an acidic intermediate. The next step has been considered as the removal of a hydride ion from a second molecule of aldehyde as follows:



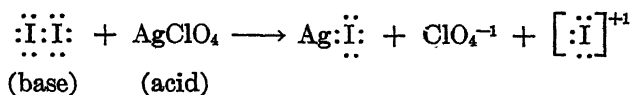
On the basis of the Lewis theory a more logical explanation would seem to be a typical acid-base reaction. The electron-deficient carbon atom of the aldehyde which is coördinated with the acidic aluminum ethylate shares a pair of electrons from another aldehyde oxygen atom acting as a base, followed by an α, γ -shift of the hydride ion:



This is a striking illustration of how the consistent application of the Lewis acid-base concepts leads to a more logical interpretation of reaction mechanisms.

Other acidic compounds such as antimony pentachloride, stannic chloride, and iodine can catalyze the reaction through the hetero-fission of the bromine molecule and the formation of the polar complex.

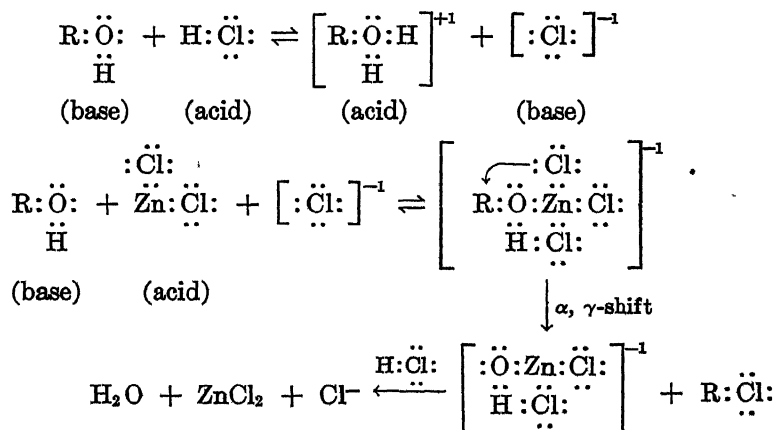
Benzene can be iodinated under anhydrous conditions by the use of silver perchlorate as a catalyst (4, 5, 6). One suggested mechanism is:



Such a simple ionic equation is probably inadequate because of the high ionic association observed in benzene (52, 92). However, there is no doubt that silver ion is acidic, as shown by its behavior in forming complexes in water and by its effect on indicators in other solvents (44).

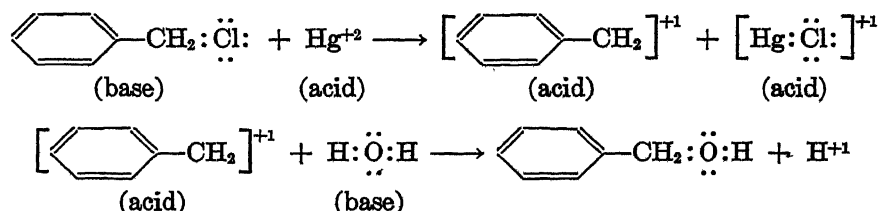
2. Alcohols

The catalytic action of zinc chloride on the conversion of alcohols to halides is well known. The catalyst used is an acid which coördinates with the basic alcohol. An α, γ -shift of the chlorine atom accounts for the formation of the organic halide (50).



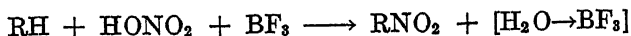
E. HYDROLYSIS

The hydrolysis of benzyl chloride is catalyzed by the presence of Hg^{+2} salts (74). The electrophilic Hg^{+2} salts are here behaving as acids and react with the organic halide to form a carbonium ion:

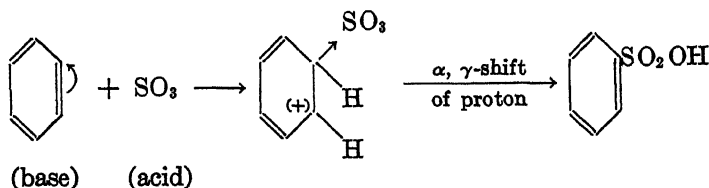


F. SULFONATION AND NITRATION

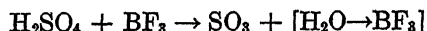
Boron trifluoride has been reported as an acid catalyst in sulfonation and nitration reactions (89). The amounts of catalyst required indicated that the reaction proceeds as follows:



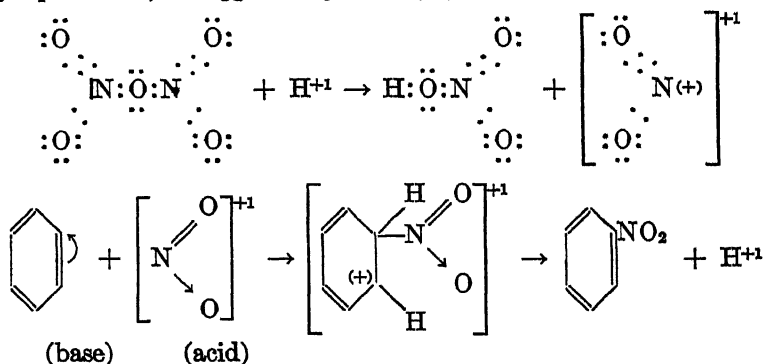
Since the rate of sulfonation increases with increasing acid concentration in sulfuric acid of less than 100 per cent concentration and with the concentration of sulfur trioxide in fuming acid (55, 66, 67), the sulfonation probably takes place largely through the typically acidic displacement of a proton by the acid SO_3 .



The catalytic function of the boron trifluoride seems to be to increase the concentration of the sulfur trioxide by combining with the water to form $[\text{H}_2\text{O} \rightarrow \text{BF}_3]$

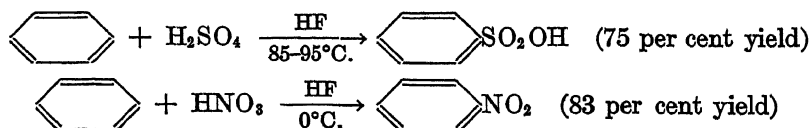


The actual nitrating mechanism is not known, but it may possibly involve nitrogen pentoxide, as suggested by Price (71).



The catalytic effect of the boron trifluoride might be due to increasing the nitrogen pentoxide concentration by combining with the water to form the coordination compound.

Hydrogen fluoride has recently been reported as having a strong catalytic effect on sulfonation and nitration reactions (85).



G. SUMMARY

In all the examples of acid catalysis considered above the characteristic effect of the acid catalyst is the increase in concentration of the acid group involved in the reaction. In most cases this is accomplished by the familiar displacement of one acid by another. In a few cases displacement does not result, but the combination of the acid with the reacting substance merely localizes the electron deficiency on one atom, which can then act acidically. One example of this is alkylation by olefins.

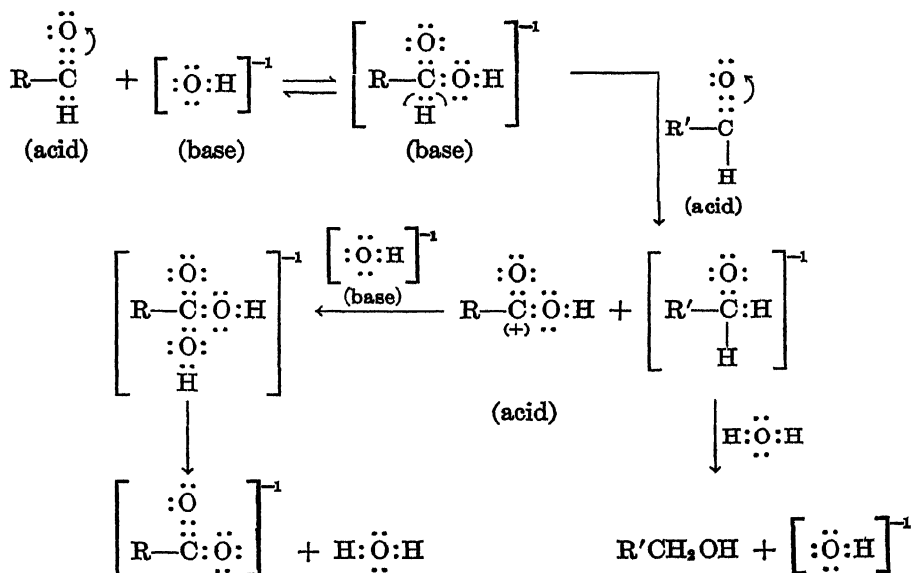
Either way, the net result is the characteristic increase in the concentration of the acid group. The effect is the same whether the catalyst is an H-acid or any other acid.

III. BASE CATALYSIS

The examples of basic substances used as catalysts in the following illustrations do not differ fundamentally from those considered as illustrations of the Brønsted concept of a base. Admittedly, these substances are proton acceptors, *but* they can and do react with acid substances other than protons. The proton is only one of many acid groups or compounds having similar electrophilic characteristics. Brønsted's bases are proton acceptors because they can furnish lone pairs of electrons to form a coordinate bond with an acid whether or not the acid is a proton, a hydronium ion, a neutral H-acid, or any other electron-pair acceptor.

A. CANNIZZARO REACTION

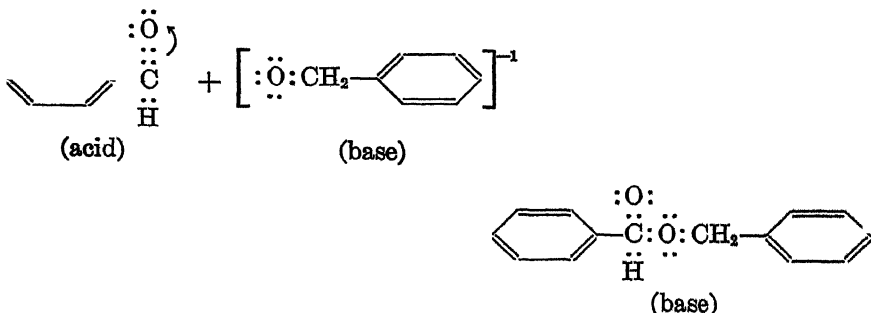
Fundamentally, the base-catalyzed Cannizzaro reaction involves the reaction between two molecules of an aldehyde containing no α -hydrogen atoms. The over-all result is the transfer of one aldehyde hydrogen to another aldehyde group, thus producing two radicals that could combine to form an ester. In most cases the presence of the basic catalyst prevents the ester formation and there results an alcohol and a metal carboxylate (19, 24, 68). The mechanism can be shown as follows:



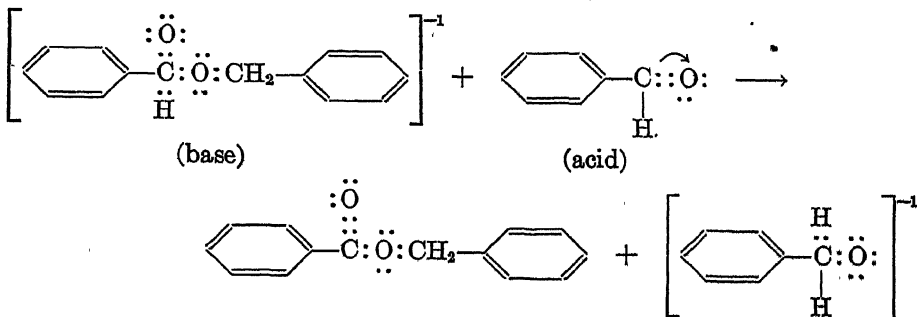
The amphoteric nature of benzaldehyde is shown by this base-catalyzed reaction and by the previously discussed acid-catalyzed reaction to form an ester. In the former the carbonyl oxygen offers a share in an electron-pair to the acid catalyst, and in the latter the carbonyl carbon accepts a share in an electron-pair from the basic catalyst. In the base-catalyzed reactions of aldehydes containing no α -hydrogen the effect of the entering basic catalyst is to shift the carbon-hydrogen electron-pair toward the hydrogen, thus favoring $\overset{+}{\text{C}}-\overset{-}{\text{H}}$ rather than $\overset{-}{\text{C}}-\overset{+}{\text{H}}$. This is true only with electron release groups such as OH^{-1} and $\text{OC}_2\text{H}_5^{-1}$ ions. This change enables a hydride ion to be split off more easily than would be the case if the basic catalyst were an electron-withdrawal group, such as the cyanide ion. This latter group would cause the carbon-hydrogen electron-pair to shift $\overset{-}{\text{C}}-\overset{+}{\text{H}}$ rather than $\overset{+}{\text{C}}-\overset{-}{\text{H}}$ and thus favor the removal of a proton rather than a hydride ion. This might explain the specificity of the cyanide ion as a basic catalyst in the benzoin condensation, which will be discussed later.

The reaction in deuterium water (21) results in an alcohol containing no deuterium hydrogen attached to the carbon. This indicates that the transfer of a hydrogen from one aldehyde molecule to another does not involve water in the transfer mechanism.

Another example of the acidic behavior of the amphoteric aldehyde and the basic action of a catalyst in this type of reaction is the formation of benzyl benzoate under anhydrous conditions.

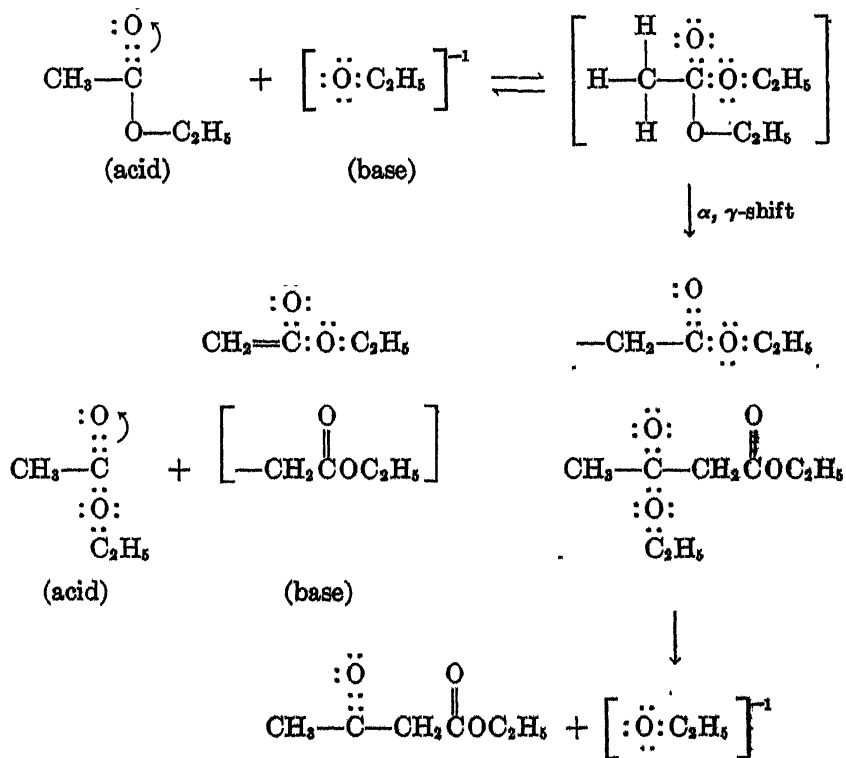


A second molecule of aldehyde reacts as an acid with this intermediate and removes a hydride ion, thus forming the neutral ester molecule and at the same time regenerating the basic catalyst, i.e., the benzyolate ion:



B. THE CLAISEN REACTION

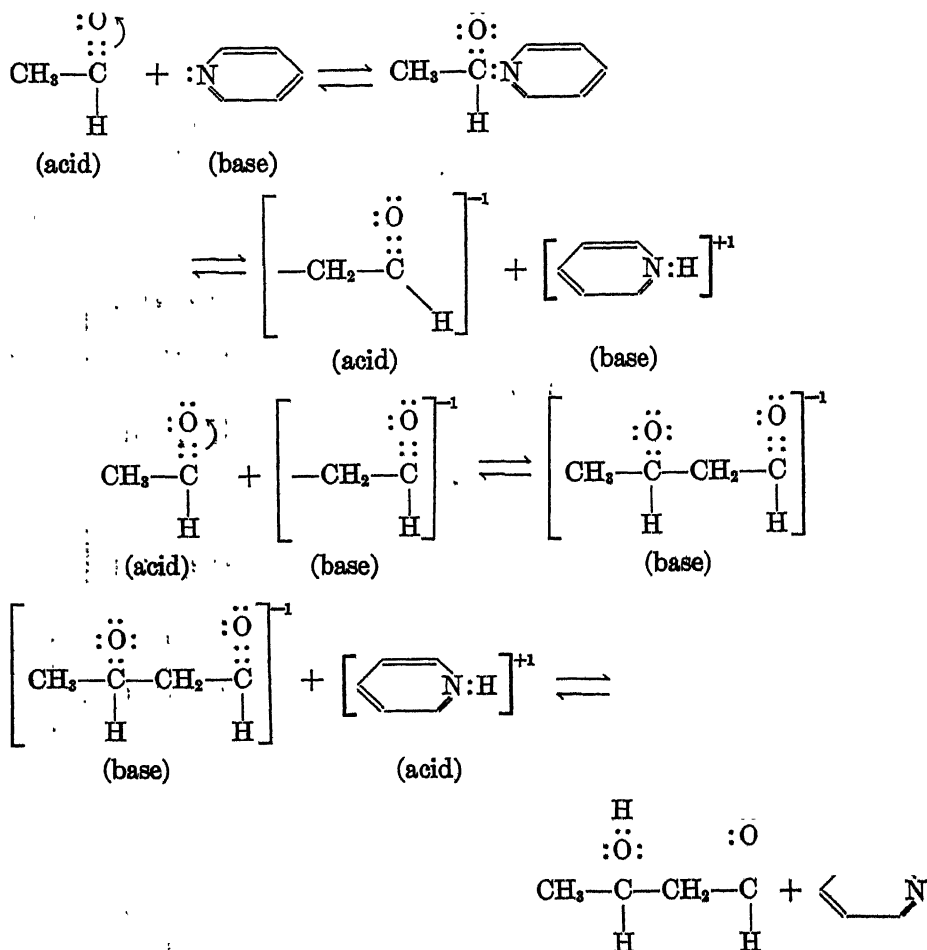
The Claisen reaction (1, 26, 27, 88) probably involves an ionic mechanism, the first step of which is an acid-base exchange in the presence of the basic ethylate ion. This exchange probably takes place through the formation of an intermediate coördinate complex, followed by an α, γ -shift and ionization to form the ester anion.



Evidence of anion ester formation according to the reactions given has been obtained (61, 75). Other bases can be used as catalysts in these condensations. The use of the triphenylmethyl carbanion has been reported in recent years (28, 30, 61, 75).

C. ALDOL CONDENSATIONS

The synthetic importance of these condensations is known to all organic chemists. A wide variety of compounds can be prepared through the condensation of two carbonyl compounds. The catalysts used are bases such as acetates, carbonates, pyridine, and amines (3). These catalytic agents are bases according to both the Lewis and the Brönsted theories. The mechanism of the reaction is represented as follows:



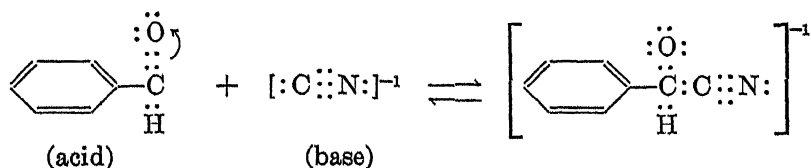
When the reaction is carried out in deuterium water (9) no deuterium hydrogen becomes attached to the carbon, indicating that water takes no direct part in the mechanism of the reaction.

The formation of diacetone alcohol has been carefully studied (38, 41). The reaction is catalyzed by ammonia, primary amines, and secondary amines (94).

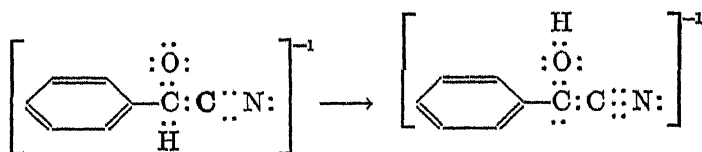
D. THE BENZOIN CONDENSATION

The specificity of the basic cyanide ion used as a catalyst in the formation of benzoïn has been considered puzzling and apparently no adequate explanation has yet been suggested (25). Hydrogen cyanide, mercuric cyanide, and sodium hydroxide have no effect on the rate of benzoïn formation, while sodium cyanide, potassium cyanide, and barium cyanide have a powerful catalytic effect (42).

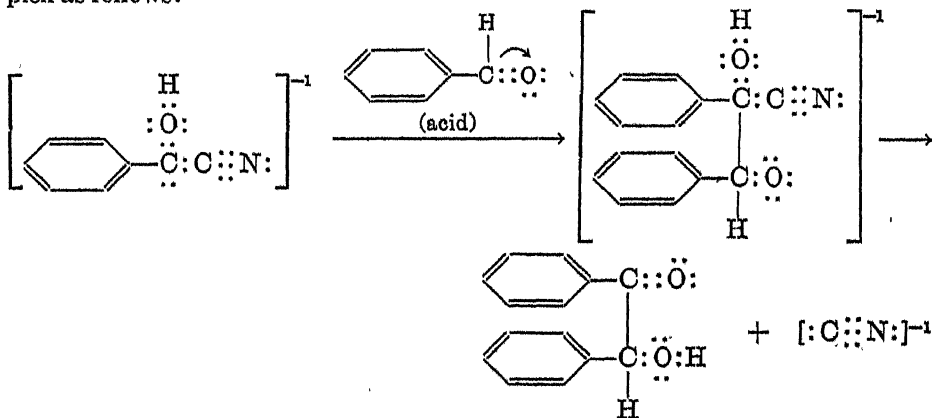
The kinetics of the reaction, however, leave little doubt that the following mechanism exists:



In the product formed the cyanide group, which is an electron-withdrawal group, greatly activates the α -hydrogen by causing an electron displacement in the C—H bond which results in $\overset{+}{\text{C}}\text{—}\overset{-}{\text{H}}$ rather than $\overset{+}{\text{C}}\text{—}\overset{-}{\text{H}}$. Therefore, instead of a hydride ion, a proton is split off by the oxygen of a similar molecule. The net result can be considered as a shift of a proton from the carbon to the oxygen of the carbonyl group. It seems reasonable to suspect that the specificity of the cyanide ion as a catalyst for this reaction is due to the fact that it has electron-withdrawal properties. The other common basic catalysts, such as OH^{-1} , $\text{OC}_2\text{H}_5^{-1}$, and the amines, all have electron-release properties, which would cause the electron shift to $\overset{+}{\text{C}}\text{—}\overset{-}{\text{H}}$ and thus produce a hydride ion rather than a proton.



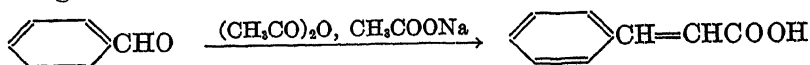
A second molecule of the benzaldehyde reacts acidically with this basic complex as follows:



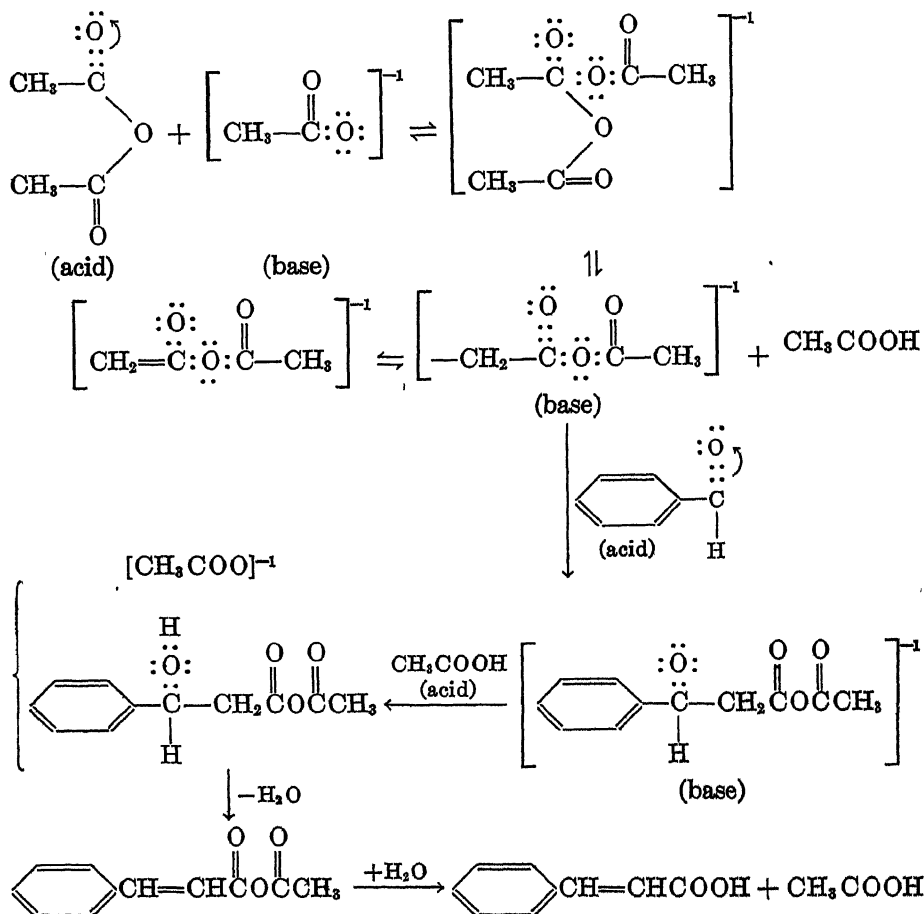
E. THE PERKIN REACTION

Fundamentally, this reaction involves an aldehyde, an acid anhydride, and the sodium salt of its acid. Although much controversy has existed in regard to the function of the anhydride and its salt, it is now generally accepted that the anion of the salt serves the function of a basic catalyst (35). Many other bases such as alkali-metal acetates, carbonates, sulfites, phosphates, pyridine, quinine, and triethylamine (2, 40, 60) have been used for the condensation.

Although the over-all reaction is represented as:

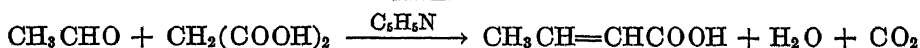
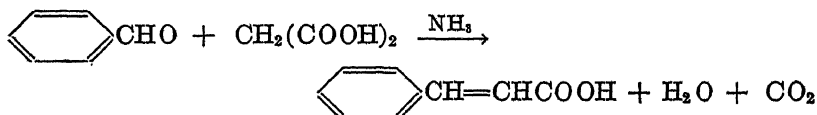


the mechanism of the reaction can be shown as follows:



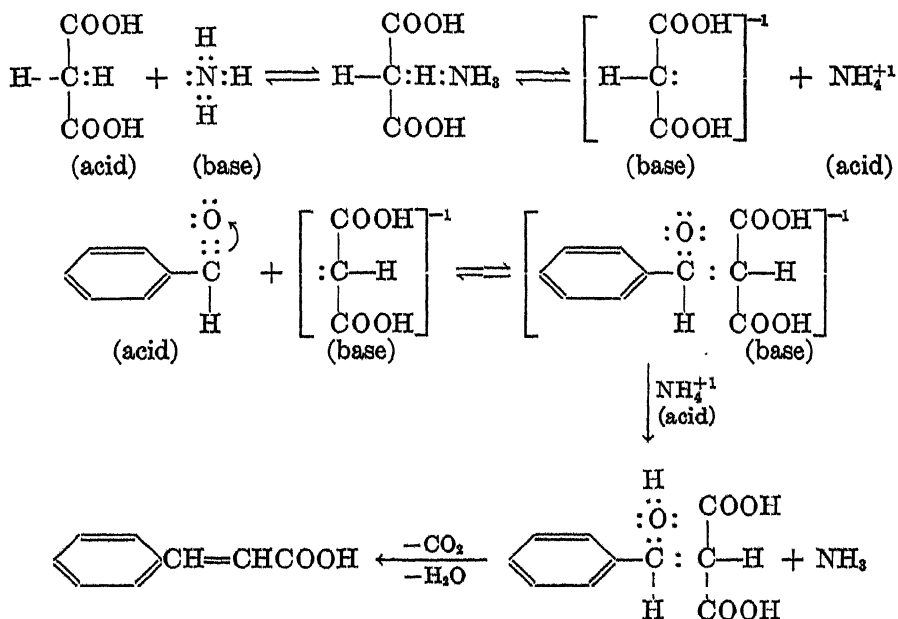
F. THE KNOEVENAGEL REACTION

Aldehydes will condense readily with malonic acid (37) in the presence of basic catalysts:



Ammonia, pyridine, primary and secondary amines, and especially piperidine, all of which are basic compounds, have been used as catalysts (12, 18, 20, 39).

The mechanism can be explained as follows:



G. SUMMARY

In all the examples of basic catalysis considered above the characteristic effect of the basic catalyst is the increase in concentration of the basic group involved in the reaction. In most cases this is done by the familiar displacement of one base by another: for example, the displacement of the ester anion from ethyl acetate by the ethylate ion catalyst in the Claisen reaction. In some cases displacement apparently does not result, but the combination of the basic catalyst with the reacting substance merely localizes the electron excess on one atom, which can then act basically. An example of this is the shifting of the electron-pair of the C—H linkage toward the hydrogen in the base-catalyzed Cannizzaro reaction, thus enabling a second molecule of aldehyde to remove a hydride ion.

IV. CONCLUSION

In presenting these examples of acid-base catalysis, no attempt has been made to survey the literature for every possible case to which the new terminology might be applied. The reactions which have been discussed were chosen primarily to show how consistently this terminology can be applied to familiar chemical behavior. In many cases the reaction mechanisms obviously can be interpreted adequately as isolated phenomena without reference to the Lewis theory. It is only as these catalytic reactions are correlated with each other and with a large body of other phenomena that any great benefit is to be expected from the application of the theory. The insight gained from such a systematic use of the theory should do much to stimulate chemical thinking and research.

The four experimental criteria of acids and bases listed by Lewis (44) are: (I)

neutralization, (II) displacement of an acid or base by a stronger acid or base, (III) titration with indicators, (IV) catalytic effects. Previous papers (44, 51) have given examples of the acid properties of many other substances besides H-acids according to the first three of these criteria. It has been shown in those papers that the behavior of substances like sulfur trioxide, boron trifluoride, aluminum chloride, stannic chloride, and silver perchlorate is analogous to the behavior of H-acids in neutralization, in displacement, and toward indicators.

The catalytic effect of these substances is well known. So in demonstrating their similarity to H-acids in this respect it is necessary to reverse the comparison made by means of the first three criteria. The behavior of H-acids must be shown to be analogous to the behavior of sulfur trioxide, boron trifluoride, aluminum chloride, stannic chloride, and silver perchlorate, rather than *vice versa*. It has been shown in the present paper that this is the case. Sulfuric acid, phosphoric acid, and hydrogen fluoride show catalytic activity similar to that of the strong non-hydrogen acids. It is apparent that the presence of a particular element is unnecessary for a substance to behave as an acid. Instead, whether hydrogen is present or not, acidity depends upon the electrophilic character of the reagent *and* upon the electrodotic character of the base.

The characteristic behavior of acids and bases toward amphoteric solvents has been discussed in a previous paper (51). If the acid is strong enough, it will increase the concentration of solvent cations. One acid displaces another. For example, sulfur trioxide displaces hydrogen ions from water. If a sufficiently strong base reacts with an amphoteric solvent, the concentration of solvent anions is increased. The base displaces the basic ion from the solvent. For example, piperidine displaces hydroxyl ions from water. In this paper it has been shown that analogous behavior is responsible for acidic and basic catalysis. Acidic catalysis is due to the increased concentration of the acidic group involved in the reaction—caused by the addition of the acid catalyst. Basic catalysis is due to a similarly increased concentration of the basic group involved in the reaction.

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THE APPLICATION OF SONIC AND ULTRASONIC WAVES IN COLLOID CHEMISTRY

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Low-frequency audible as well as high-frequency inaudible sound waves ("ultrasonics") of great intensity are able to produce a great diversity of phenomena pertaining to the realm of colloid chemistry (86). They may bring about various dispersion effects, such as the mutual emulsification of two bulk liquid phases, the disintegration of certain solids, peptization, the liquefaction of gels, the depolymerization of high-molecular substances, the formation of fogs, etc. On the other hand, they may also cause accumulation, aggregation, and coagulation; with anisometric particles, orientation in liquid as well as in gaseous systems may result. In addition, sound waves degas liquids and always heat the irradiated systems; they further favor the formation of new phases in some instances, e.g., they start crystallization from many supersaturated solutions and affect the crystalline structure of metals and alloys when applied to the melts during the period of cooling and crystallization.

There are no fundamental physical differences between sound waves of low, medium, and high frequencies; however, great differences exist in the ease of their production and transmission. Originally ultrasonics of several hundred thousand cycles per second were used exclusively for colloid work; later, frequencies down into the audible range have come into more general use. At very high frequencies the energy losses become excessive.

Four main groups of sound generators have been used for colloid work: (a) piezoelectric; (b) magnetostrictive; (c) electromagnetic sound generators; and (d) the gas current vibration generator, a device useful only in gaseous systems.

The *piezoelectric sound generator* transforms high-frequency electric oscillations into mechanical oscillations by making use of the piezoelectric effect. For colloid work the frequency range from 100,000 to 1,000,000 cycles a second is used.

If an alternating electrical field is applied to a piezoelectric crystal, e.g., a quartz crystal plate, the crystal contracts and expands periodically—it vibrates. The frequency of these vibrations is that of the applied electrical field. At a given field strength the vibrations become much stronger if the quartz oscillator vibrates in resonance; then electrical energy can be transformed efficiently into mechanical oscillations.

In gases the energy transmission from the solid oscillator to the surrounding medium is very poor. If, however, a quartz crystal vibrates in a liquid, then the energy transfer is good and the useful energy output may become rather high.

For most work on colloids one immerses the quartz oscillator with a suitable electrode system in a bath of oil which transmits the vibrations easily to any

other system dipped into it (2, 30, 32, 35, 43, 86). The electrical oscillations which operate the crystal are generated by an electronic oscillator with an energy output of several hundred watts. It is coupled to the electrode system by means of a Tesla coil. Figure 1 shows the general scheme of the piezoelectric genera-

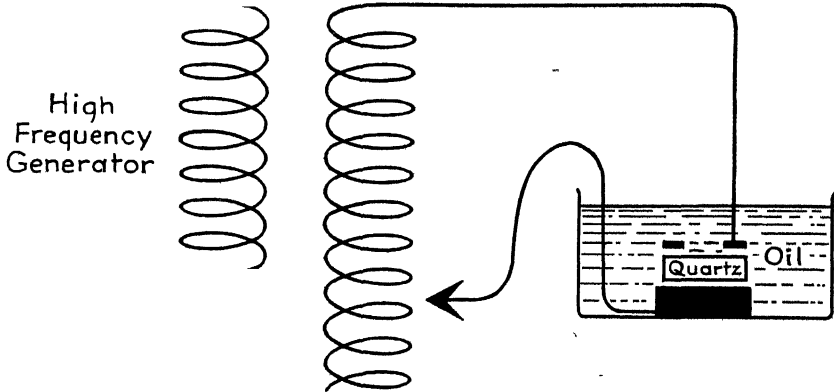


FIG. 1. Piezoelectric sound generator

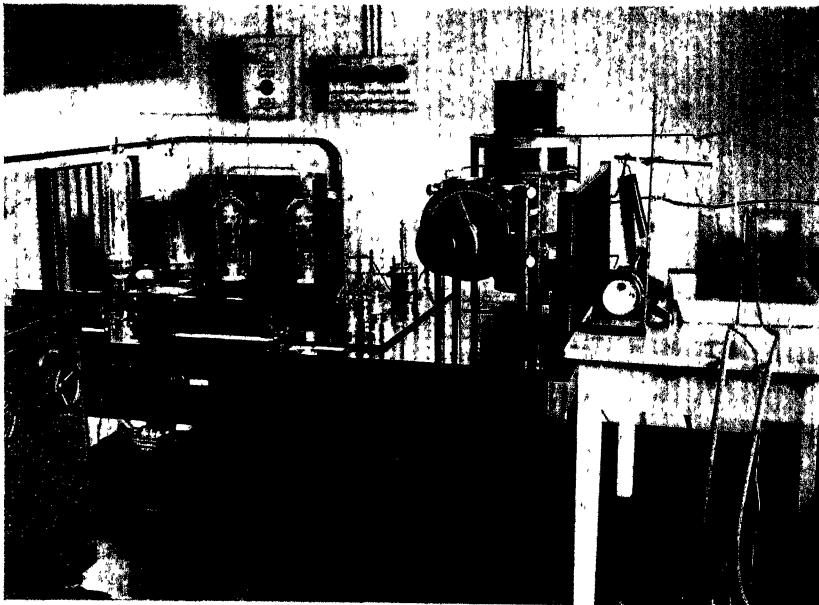


FIG. 2. Laboratory model of piezoelectric sound generator (from K. Sollner)

tors; figure 2 is a photograph of the apparatus used by the author; figure 3 shows a quartz oscillator and an electrode system in an oil bath, as used, e.g., for emulsification.

The *magnetostriction generator* transforms alternating electric currents into sound vibrations, making use of the magnetostriction effect (61).

A rod or tube of ferromagnetic material brought into a magnetic field parallel to its axis decreases or increases in length, depending upon the nature of the material, its previous treatment, the applied field strength, and other factors. This effect is known as "magnetostriction."

In an alternating magnetic field the length of the rod changes periodically by magnetization. Resonance between the natural elastic period of the rod and the frequency of oscillations caused by the magnetic field increases the efficiency of the energy transformation greatly.

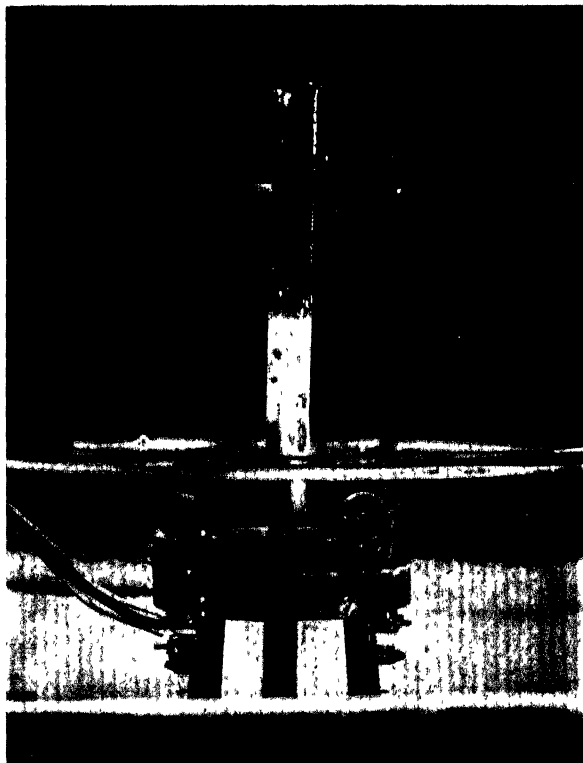


FIG. 3. Quartz oscillator and electrode system of a piezoelectric sound generator in an oil bath as used for emulsification (from K. Sollner).

The alternating magnetic field used in the magnetostriction sound generators is produced by an alternating electric current (14, 15, 16, 26). Figure 4 shows the construction of a laboratory model described by Chambers and Flosdorf (14).

Such generators are used in liquid as well as in gaseous systems; in the latter case a large piston-like end-plate affixed to the vibrating tube facilitates the transmission of energy into the gaseous phase. The range of useful application is from several thousand up to 50,000 cycles a second. The corresponding relatively great length of the sound waves is one of the main disadvantages of these generators for work in liquid systems. Their advantages are their rela-

tively simple and cheap construction and the fact that the oscillator itself—usually a vibrating nickel tube—may in most cases be brought in direct contact with the substances to be treated without the interposition of another medium. It seems likely that magnetostriction generators will at least partially displace piezoelectric oscillators for colloid work.

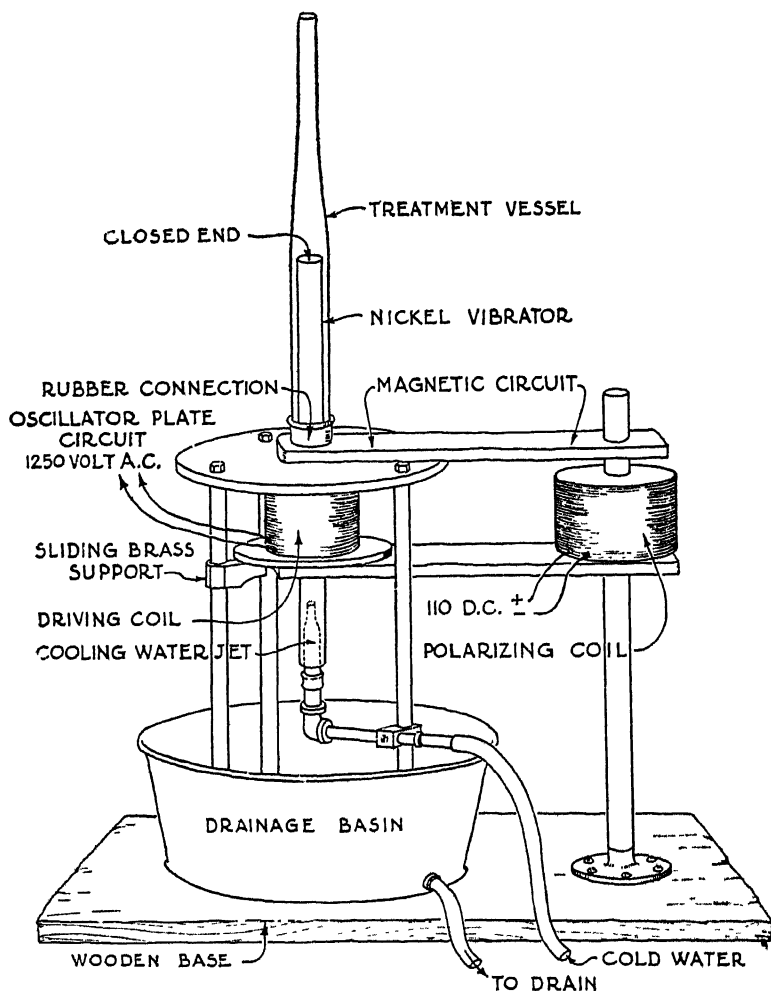


FIG. 4. Magnetostriction sound generator (from L. A. Chambers and E. W. Flosdorf); reproduced by permission.

The *electromagnetic sound generators* have so far been used in only a few cases. Their frequency is entirely in the lower audible range.

Chambers (10) describes two types of oscillators, one of conventional electromagnetic design, and one of the Fessenden type. The electromagnetic oscillators are similar to those used in submarine communication and echo depth sounding; they consist essentially of a heavy steel membrane actuated by

alternation in an electromagnetic circuit. The oscillators of the Fessenden type have the vibrating membrane fixed to a copper tube, the whole being polarized by an alternating inciting current. The movement of the diaphragm depends on reversal of eddy currents in the copper tube induced by current oscillations in an activating coil. Clair (16) developed a generator for the study of the coagulation of smoke; it is claimed to be very efficient. All these devices are operated at their natural resonance frequency.

Since only a few investigators have used electromagnetic sound generators, it is difficult at present to appraise their merits justly. They are very promising in the treatment of gaseous systems. If it should be found possible to operate electromagnetic sound generators really efficiently in liquid systems, it seems likely that this method could find wide industrial application.

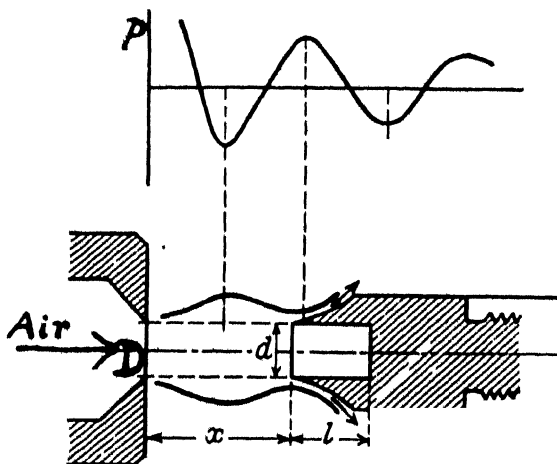


FIG. 5. Gas-current sound generator

Gas-current vibration generators are designed for attaining great energies of vibration in gases (Hartmann (33)). If a current of air is allowed to issue from a nozzle at a speed greater than sound velocity, a periodic structure is formed in the air stream. The pressure, P , varies periodically at different distances from the mouth of the nozzle, D , as indicated in the upper half of figure 5. This makes possible the production of sound waves when a hollow body, serving as an oscillator, is brought into these regions of instability (figure 5, lower half). The frequencies of the sound waves are determined by the dimensions l and d of the hollow oscillator, and its distance x from the nozzle D . Thus one is able to produce oscillations ranging from infra-acoustic up into high ultrasonic frequencies. The gas-current vibration generator may find an important application in the coagulation of industrial smokes and fogs.

II

Emulsification occurs if a test tube containing water and mercury, or water and an immiscible organic liquid, is dipped into the vibrating oil of a piezoelectric

sound generator, or if the same two-phase systems come in contact with the vibrating nickel rod of a magnetostrictive sound generator. Grey clouds of very fine mercury droplets are thrown into the water from the water-mercury interface; in oil-water systems white clouds of dispersed water and organic liquid, respectively, are produced where the two liquids meet and soon more or less concentrated emulsions are formed (compare figure 3). In small-scale experiments 6 g. of mercury per liter and 60 g. per liter of benzene or similar substances may be dispersed in 1 or 2 min.; in the absence of any protecting or stabilizing agent these concentrations do not increase on further irradiation. In the presence of suitable emulsifiers very high concentrations can be obtained.

The mechanism of dispersion of liquid and molten metals according to Richards (65; see also 78) is as follows: When the vessel containing the metal and the water is irradiated, violent transverse vibrations are set up in the walls of the



FIG. 6. Emulsification of a metal by ultrasonics: a low-melting alloy solidified during irradiation. About twice actual size (From K. Sollner)

vessel and small quantities of water are pumped into the liquid metal; the water droplets rise through it and reach the interface metal-water covered with a thin film of metal. This film breaks and a cloud of minute metal droplets is thrown into the water. This obviously occurs also between the water droplets in the interior of the liquid metal. A low-melting alloy cooled during irradiation, therefore, as shown by Sollner (76), is sponge-like (figure 6). This mechanism of the formation of mercury sols by sound waves is basically the same as by normal mechanical dispersion (Nordlund (55)).

Harvey (35) showed that in non-metallic systems emulsification occurs neither *in vacuo* nor when sufficiently high outside pressure is applied. This rule applied even in the presence of the best emulsifiers (77). When the system is in equilibrium with a gas phase, emulsification always occurs on irradiation, provided this pressure is neither too high nor too low. This very striking effect of the gas pressure is found without exception (76). According to Chambers and Gaines

(15) and Bondy and Sollner (77), the mechanism of emulsification in non-metallic systems is as follows: A sound wave travelling through a liquid compresses and stretches it periodically. If the stretch is moderate and the irradiated liquid is free of gas, nothing spectacular occurs; but if the liquid is stretched unduly, even most carefully degassed liquids show zones of a slight and somewhat glittering opacity but no bubbles rise to the surface. A hissing noise is always heard under these conditions (15, 52, 74, 77).

The glittering opacity indicates that the liquid disrupts and forms cavities under the stretch of the sound waves. High hydrostatic pressure prevents this disruption, and no hissing noise can be observed. *In vacuo*, even at low energies, the liquid bubbles and boils, and no hissing noise is heard. Under neither of these conditions does emulsification occur. Strong mechanical action caused by ultrasonics is always accompanied by a hissing noise. Obviously the formation of cavities alone does not cause emulsification; their disappearance under pressure, which is accompanied by a hissing sound, brings about the formation of emulsions (15, 74, 77).

The enormous local stresses and accelerations which occur when a vapor bubble collapses in a liquid (Rayleigh (63)) cause the intense mechanical effects. The entire phenomenon involving the formation of cavities and their violent collapse due to outside pressure is called "*cavitation*." Cavitation occurs in an irradiated system preferentially at weak spots, where the energy of disruption is relatively low, as at oil-water interfaces (74, 77). We may visualize cavitation which leads to emulsification as a very violent hammering of the collapsing cavities, occurring at the phase boundary.

Before discussing emulsification and emulsions further, it seems appropriate to mention one more effect caused by the stretching of liquids and by cavitation. In liquids which are saturated with gas, cavitation is always accompanied by partial *degassing* (23). When the liquid is stretched and cavities are formed, some of the dissolved gas is obviously released into the latter; the cavities cannot collapse completely. Gas bubbles remain which, as we shall see below, unite readily under the influence of the sound waves into bigger ones and rise to the surface. Actually true cavitation, as described above, is not necessary for degassing; much weaker sound intensities are effective. Boyle (3, 4) is inclined to attribute this degassing to the presence of preformed gas nuclei; however, it seems more likely that the mechanism is as follows: When a sound wave passes through the liquid, the latter is stretched periodically; in this period it is supersaturated with respect to the gas, and minute gas bubbles are formed and coagulated.

We must break off the discussion of the degassing effect, which needs further careful experimental and theoretical investigation, and return to the problem of emulsification and *emulsions*.

Besides mercury, other metals may easily be dispersed if their melting points are low enough. They are dispersed only in the liquid state. In many cases stable truly colloidal solutions are obtained (81, 86). Solid metals cannot be dispersed merely by sonic irradiation.

Oil-in-water and water-in-oil emulsions can apparently be produced with any pair of incompletely miscible liquids, the limiting concentrations depending upon the nature of the system (79). In properly protected systems 75 per cent emulsions of oil in water are readily obtained.

The sonic methods of emulsification are undoubtedly very easy, convenient, and reproducible in studying emulsification and emulsions. Not the least of the advantages is that very small samples, as small as a drop, may be emulsified,—if necessary in sealed containers. On the other hand, large-scale operation is possible; Chambers (10, 11) has homogenized milk to induce soft curd character. With his electromagnetic sound generator and with a power input of 1 to 2 kilowatts, 250 gallons of milk per hour were treated successfully; with a magnetostriction sound generator at much lower rates of flow partial sterilization was also observed.

Mention must be made here also of the work by Masing and Ritzau (54), Schmid and Ehret (68), and others. *Dispersions of one immiscible metal in another* were prepared (lead in aluminum, calcium in silicon, etc.) by the application of intense sound waves to melts. The mechanism causing these dispersions is probably similar to the dispersion of mercury in water. It is claimed that these metal-metal dispersions have in the cooled state some highly desirable metallurgical properties, which are otherwise unobtainable. Further work along these lines seems to be highly desirable from the practical point of view.

The *formation of fogs*, i.e., the “atomization” of non-metallic liquids by ultrasonics (Wood and Loomis (86)) is best shown by irradiating a beaker containing some fairly volatile liquid with sound waves of high intensity. The beaker fills rapidly with a white cloud. With more viscous liquids special experimental arrangements must be used. The formation of fogs is due to the same mechanism as that causing emulsification in non-metallic systems, i.e., cavitation (73).

The *peptizing action of sound waves* on gels, gel-like substances, precipitates, and sediments has been extensively investigated. All such systems contain preformed particles of colloidal or semi-colloidal size. In all the numerous instances investigated so far it has been found that the primary particles may be separated from one another readily by cavitation caused by audible or inaudible sound waves (28). The preparations made by sound action do not show any special features; the sound waves act merely as a very efficient mechanical dispersing device.

The sound method of dispersion and peptization of sediments, precipitates, and gels undoubtedly offers many advantages on account of the cleanliness and good reproducibility of this method and the small quantities of materials necessary.

A case of special practical interest is the preparation of extremely fine-grained photographic emulsions of great sensitivity. Claus (17) and Dangers (20) irradiated silver bromide emulsions during their preparation. Claus reported in detail on the properties of these photographic preparations. The mechanism of the action of the sound waves in this case is not definitely known. One would be inclined to attribute it to cavitation. However, other suggestions have been

made: the thermal effects associated with strong sound action (to be discussed below) and chemical activation (see below) (e.g., 62).

A *dispersion of solids in liquids* by sound methods can only be achieved with substances of moderate cohesion which are easily split and broken. This dispersion is due to cavitation. Materials comparatively easily dispersed in water, yielding colloidal or semi-colloidal solutions, are (crystals of) mica, gypsum, steatite, hematite, sulfur, and graphite (75). Some reports in the literature which claim the dispersion of metals and other substances of great cohesion have been shown to be based on faulty experiments.

The solid substance to be dispersed need not necessarily be of macroscopic size; even the particles of truly colloidal solutions may be broken down further by irradiation (75).

In order to circumvent in the case of metals the difficulties inherent in the dispersion of solids of great cohesion, Claus (18) and Claus and Schmidt (19; see also 84) submit systems to the action of strong ultrasonic waves in which metallic precipitates are simultaneously formed by electrolysis or chemical reaction. As cathode in the electrolysis process a non-corrosive metal, such as stainless steel, is used, to which the products of the electrolysis adhere poorly. The freshly formed metal is torn off the electrode (presumably by cavitation) and thus dispersed in the liquid. The authors cited report that they produced in this manner fine-grained dispersions of platinum, gold, silver, mercury, copper, cadmium, lead, bismuth nickel, chromium, iron, and aluminum. (The dispersions of the less noble metals are probably not of a purely "metallic" character.) The mercury emulsions produced by this method were much finer grained than those produced by dispersion of the bulk phase without electrolysis (19). There is little doubt that this method could be used to great advantage in many practical problems.

There are other electrode effects caused by sonic irradiation. Foremost among them is the *neutralization of passivity, and changes in electrode potentials*, both probably caused by the destruction of protecting layers.

In considering potentially useful applications of sonic dispersion, the straightforward *sonic extraction* method of Chambers and Flosdorf (14) for the preparation of labile bacterial constituents should be mentioned. By intense sonic irradiation bacteria in suspension are broken up and finally disintegrated completely (86); labile substances which are thereby released can thus be prepared in many cases, apparently without deterioration in their biological activity (14).

Changes in the anomalous viscosity of colloidal solutions, the splitting of macromolecules, and related phenomena caused by sound waves have been described frequently. One of the simplest and most easily understood cases is the reversible liquefaction of thixotropic gels (28, 30), the primary particles of these gels being loosened by cavitation. The liquefaction of thixotropic gels can be considered as an extreme case of change in anomalous ("structural") viscosity.

"Fluid" systems, the viscosity of which is decreased by sound waves, were first described by Szent-Györgi (83) and Szalay (83) (gelatin, starch, gum arabic). More thorough investigations of this problem were carried out by

Freundlich and Gillings (25) (gum tragacanth, gum arabic, gelatin, agar, cotton yellow, benzopurpurin, vanadium pentoxide, sodium stearate, and sodium oleate), and by Schmid and collaborators (67) (polystyrene, polyvinyl acetate, polyacrylic acid esters, nitrocellulose, rubber, etc.) (see also 21, 41, 66).

The solutions of gums show the simplest behavior. By short (0.5 to 8 min.) sonic treatment their viscosity and its anomalies are reduced: they become less viscous and show smaller deviations from Poiseuille's law. This effect is due to cavitation; on standing the viscosity rises again, but full recovery does not occur (25).

With gelatin and agar solutions weak irradiation is sufficient to reduce the viscosity only at low shear rates; more intense irradiation effects an over-all reduction of viscosity at all shear rates. The solutions recover their structural properties rapidly on standing. If cavitation is rendered ineffective, the anomalous viscosity of gelatin solutions is much less affected by ultrasonic irradiation; however, some reduction in viscosity still occurs. Freundlich and Gillings think that in the region of lower shear rates the resistance of the solutions to flow is probably due to gel formation; at the higher shear rates we have a closer approach to a viscous resistance, the gel structure is destroyed, and the behavior is similar to that of a non-gelating solution, like a gum. Schmid and collaborators found a similar complexity of behavior in several other cases; they suggest that frictional forces resulting from the relative motions of a rigid network of macromolecules and the vibrating solvent may cause the breakdown of macromolecules (67). The degree and rate of recovery in such systems should be studied further; this problem blends gradually with that of influencing polymerization reactions.

Solutions of cotton yellow show distinct anomalous viscosity and strong streaming double refraction due to the presence of ultramicroscopic rod-shaped particles. Brief, low-intensity irradiation completely destroys (by cavitation) this anomalous viscosity, and the strong stream double refraction likewise disappears. There is but little recovery on standing. Ultramicroscopic examination after irradiation shows that the rod-shaped particles have disappeared, very fine, seemingly isotropic particles being present instead (25).

The viscosity of sodium stearate solutions is reduced rapidly by cavitation caused by intense ultrasonic irradiation; this is accompanied by a most marked change in appearance. Whereas the original solution has a curdy, opaque, somewhat gelated, but generally homogeneous appearance, the system after irradiation is a limpid suspension of silky, needle-shaped, crystalline particles, showing streaks of stirring. Ultrasonics in this case accelerate crystallization.

By irradiation with ultrasonics Brohult (8) has split hemocyanin into fragments of one-half and one-eighth their original size. Confirmation and expansion of this highly interesting observation seems most desirable. The denaturation of certain proteins by the action of sound (13, 53) in many cases seems to be due to a kind of surface denaturation.

III

After this outline of the disruptive and destructive effects of sonic and supersonic waves, which in most cases are caused by cavitation, we may turn now to their *ponderomotive action* and the phenomena which depend upon the latter.

The best-known ponderomotive effect of sound waves is the *accumulation* of fine solid particles in the nodes of a field of stationary sound waves in *gaseous systems*—Kundt's dust figures (51). The phenomenon is so well-known that a mere reference to figure 7 seems sufficient. In a sound field of ultrasonic frequency the phenomenon is much the same,—fine dust collects in the nodes.



FIG. 7. Kundt's dust figures in gases

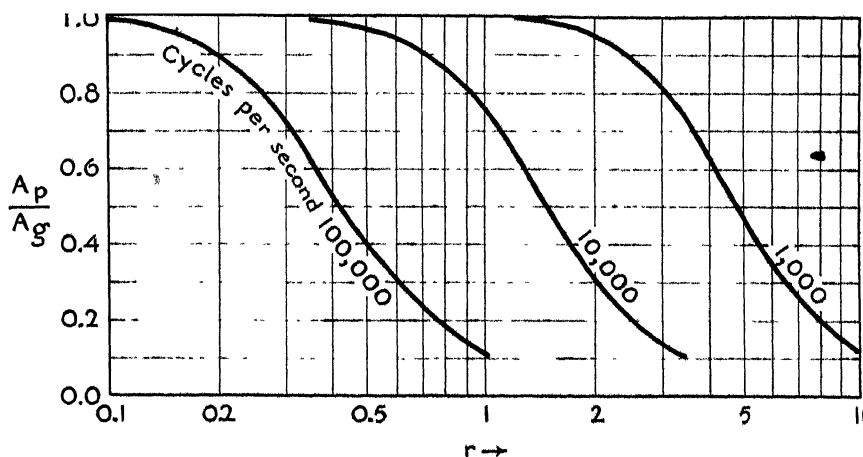


FIG. 8. The ratio of the amplitudes A_p/A_g in its dependence on particle radius (in microns) for different frequencies. (Curves calculated by H. Freund and E. Heidemann)

The movements of the particles of an aerosol in a sound field are too complicated to be discussed quantitatively here. Suffice it to say that the individual particles of an aerosol follow the oscillations of the gaseous medium the more closely (1) the lower the frequency, (2) the smaller the mass of the particle, (3) the lower its density, and (4) the higher the viscosity of the gaseous medium. Small, light particles follow the movements of the medium almost completely at low frequencies; at higher frequencies this movement decreases as the frequency increases. Large particles oscillate but little even at low frequencies, their inertia being great compared with the forces acting upon them; they are practically stationary at high frequencies. Very instructive photographs of particles oscillating in a sound field were published by Brandt, Freund, and Heidemann (46). In the diagram of figure 8 are plotted the ratios of amplitudes

of particle and gaseous carrier, A_p/A_g as a function of the particle radius, r , for these different frequencies, as calculated by Brandt, Freund, and Hiedemann (46). It is evident that with smoke and fog particles of the sizes that usually occur in natural and artificial smokes and fogs (0.5 to 5μ), the strongest effects can be expected in the region of sonic and lower ultrasonic frequencies.

The impulses received by the oscillating particles toward a node and away from it are not strictly symmetrical. The impulses from the side of the loops are slightly larger, since the loops are the places of the maximum movement of the oscillating gas. The net result of this asymmetry of oscillation is that the particles are slowly shifted toward the nodes, where they accumulate and aggregate (coagulate).

This *coagulation of aerosols* is without doubt the most intensively investigated action of sound waves in colloidal or semi-colloidal systems. Sonic coagulation in aerosols has been discussed by Patterson and Cawood (59), Pearson (60), Andrade and Parker (1, 58), Gottschalk and Clair (31), Clair (16), Brandt, Freund, and Hiedemann (6, 7, 42, 43, 44, 45, 46, 47), and many others.

In fogs and smokes practically all collisions between particles are non-elastic and lead to the formation of aggregates, i.e., they coagulate spontaneously. In view of the described oscillations and the accumulation of aerosol particles in a sound field it is not surprising that the coagulation of aerosols can be greatly accelerated if they are exposed to sound waves of higher acoustic or lower ultrasonic frequency.

The usual experimental set-up consists substantially of a cylindrical gas chamber, which is either filled with the aerosol, or, more advantageously, through which the aerosol flows continuously. The system is adjustable so that a stationary wave field may be set up; then the intensity of the sound field becomes many times that obtainable without this precaution, and rapid coagulation sets in, even in fast-flowing aerosols. Whereas under the proper conditions the untreated aerosols settle down as fine dust, mainly as primary particles, the sound-treated aerosols yield sediments composed of large aggregates. In many cases aggregation to a mass several thousand times that of the primary particles has been observed.

A quantitative theory of sonic coagulation is confronted with the greatest difficulties. In the process of coagulation, aerosols become very heterodisperse and the aggregates so formed deviate widely from a spherical shape. The worst difficulty is that the mechanism of coagulation is not simple, a number of not entirely independent factors working simultaneously to produce aggregation. The two main factors are hydrodynamic attraction between the oscillating particles and the increased probability of kinetic collisions between the particles oscillating with different amplitudes; the latter factor, as pointed out by Sollner (72), can be considered as a special case of orthokinetic coagulation (85). In addition, there are the changes in concentration and the spontaneous sedimentation of the aggregated particles which leads to orthokinetic coagulation, as originally conceived by Wiegner and collaborators (85) for liquid systems. The problem is so complex that a further discussion in this brief outline is impossible. The most up-to-date review has been given by Hiedemann (43).

Numerous attempts have been made to apply sonic coagulation methods to the precipitation of industrial fogs and smokes. Since coagulation occurs readily in streaming aerosols it is possible to handle large volumes; however, on the basis of the available literature no definite statement can be made at present as to whether or not sonic coagulation may be developed into an economical industrial method; the chances that this may be achieved seem to be rather good.

Accumulation in liquid systems: As in gaseous systems, accumulation occurs in liquids with dispersed gases, solids, and liquids, as described by Boyle *et al.* (4, 5). Sollner and Bondy (80) have investigated this accumulation more closely from the colloid chemical viewpoint. Stationary waves cause the formation of very beautiful and regular patterns—Kundt's dust figures (figure 9)—in liquids (76). With increasing time of irradiation the zones of accumulation may become very sharp; under favorable conditions thin discs with well-defined boundaries are seen in the liquid. The distance between two such bands is $\lambda/2$, λ being the wave length in the liquid. So far as experimental evidence shows at present,



FIG. 9. Arrangement for the study of stationary wave patterns. About one-fourth actual size. (From K. Sollner and C. Bondy)

accumulation occurs at the nodes if the dispersed substance is less dense than the medium of dispersion, and at the antinodes if it is denser. In systems containing particles both denser and less dense than the dispersion medium, sound waves may separate the two kinds of particles from each other, the distance between a band of the one and the other being $\lambda/4$ (80).

The rate of accumulation of dispersed substances at the nodes or antinodes depends on the size of the particles; very sharp wave patterns are formed immediately with particles of 4 to 10 μ . With particles of 1 μ and smaller, zones of diminished concentration appear after several seconds, but even after 30 min. the whole pattern is blurred. With particles of less than 0.5 μ , the effect is still less distinct. In truly colloidal solutions accumulation has not been obtained at all by Sollner and Bondy with a frequency of 214,000 cycles per second (80).

The theory of the ponderomotive action of sound waves has been discussed by Boyle (4, 5) and King (48); it is too complicated to be considered here.

Very closely linked to the accumulation effect is the *orientation of anisometric*

particles in liquid systems. Sollner and Burger (82) found that anisometric particles are oriented in a sound field with their long axes perpendicular to the flux of energy. The phenomenon is best observed in a field of stationary waves. If a dilute suspension of mica, mosaic gold, certain clays, or similar materials is irradiated, silky, brilliantly glittering zones at half-wave distance appear immediately, a sure sign of orientation. The energy necessary to bring about orientation is very much smaller than that required for accumulation, the movement necessary for the former being only of the order of the length of the particles.

As in the case of accumulation, orientation also depends on the particle size, larger particles being much more readily oriented. Orientation (contrary to accumulation) can, however, be observed with truly colloidal solutions, as has been demonstrated with aged vanadium pentoxide and ferric oxide sols (82). Hermans (39), Oka (56), and others have investigated the orientation phenomenon further, and have tried to develop the physical theory of the effect, mainly on the basis of theoretical considerations of King (48).

The *coagulating action of sound waves in liquid systems* containing macroscopic particles on the one hand and truly colloidal solutions on the other seems to be due to entirely different mechanisms; the two cases must therefore be treated separately.

The *coagulation in liquid systems with visible or microscopic particles* can be demonstrated very strikingly by irradiating unstable suspensions, e.g., quartz powder in an organic liquid. If such a suspension is irradiated even with low sound intensities, the particles accumulate and aggregate readily; the aggregates so formed soon reach a size which favors rapid sedimentation. A few seconds of weak irradiation are sufficient to clear the suspended particles from the liquid. Very similar is the coagulation of gas bubbles in liquids, whether they are pre-formed or are generated in the system by sound waves, as discussed above.

In a less spectacular manner the same result is obtained with more stable emulsions and suspensions. The coagulation always occurs in the zones of accumulation in the liquid (figures 10, 11a, and 11b). With oil emulsions cavitation must be prevented. Longer application of sound causes in emulsions not only aggregation but also coalescence; emulsions may be broken completely (figure 11b). With highly protected emulsions the particles aggregate, but coalescence occurs only extremely slowly; the aggregates may easily be redispersed. The same, of course, is true for highly charged, stable suspensions.

Qualitatively, Sollner and Bondy (80) explain the coagulating action of sound waves in systems with microscopic particles in this manner: (1) the particles are accumulated and the rate of spontaneous coagulation increases rapidly as the concentration increases; (2) particles of different size migrate with different velocities toward the zones of accumulation, thus being liable to additional collisions, a kind of "orthokinetic" coagulation (85); (3) the asymmetry of the oscillations which the particles perform according to their (different) size causes additional collisions. Whether the factors enumerated completely explain the phenomena is still open to question; it is possible that hydrodynamic attraction

between oscillating bodies (see, e.g., reference 57) in the zones of accumulation comes into play, as may also dipole forces which are believed (40) to be the most important single factor with truly colloidal solutions.

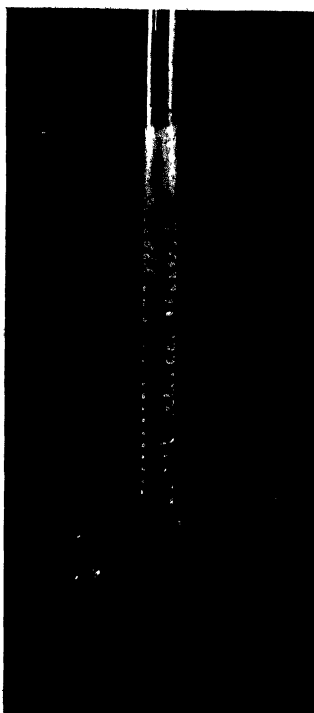


FIG. 10. Coagulation of an emulsion by ultrasonics. About one-quarter actual size. (From K. Sollner and C. Bondy)

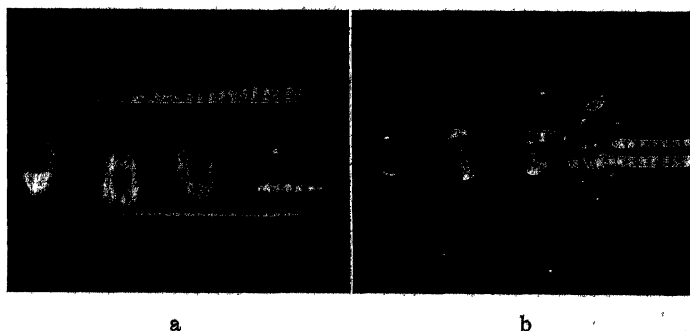


FIG. 11. Coagulation of an emulsion: the formation of oil drops in the zones of accumulation (a) after brief sonic treatment and (b) after prolonged sonic treatment. (From K. Sollner and C. Bondy)

It seems not improbable that sonic coagulation in systems with macroscopic and microscopic particles may assume some technical importance in the future. Several years ago we (30) suggested the removal of the gas bubbles from the

very viscous technical viscose solutions. The degassing of metallic melts and of glasses was tried by Krüger (50) and others (e.g., 49). In all these cases the useful process is more a coagulation of preformed gas bubbles than a true degassing. The breaking of crude oil emulsions is also mentioned in the patent literature.

Coagulation in truly colloidal solutions may be brought about by the action of sound. The mechanisms involved, however, must of necessity be of a different nature, since the ponderomotive effects responsible for the coagulation in systems with microscopic particles are practically absent, as pointed out above. Hermans has investigated the problem theoretically (38), as well as experimentally (40), with dilute silver iodide sols. The very slow rate of spontaneous coagulation in these sols is hardly influenced by sound treatment. In the presence of electrolytes—ammonium chloride, sodium nitrate, potassium nitrate (0.6–0.24 *M*), and barium chloride (0.001–0.02 *M*)—the rate of coagulation is increased in a sound field of moderate or high energy. This influence is very pronounced at low electrolyte concentrations, that is, in the range of “slow coagulation”; it is almost negligible at high electrolyte concentrations, where the coagulation is rapid. Hermans thinks that neither heat effects, the kinetic energy of oscillations, nor orthokinetic coagulation, accumulation, nor hydrodynamic interaction between the particles can account for the observed facts. He sees the most promising explanation in the dipolar interaction of the oscillating particles (“migrating dipoles”), which results from the distortion of the electric double layer surrounding the individual particles. This problem seems to be still far from a definite solution; much more experimental work with a variety of sols is needed.

Closely related to the phenomenon of accumulation in systems with microscopic particles is the acceleration of *solidification* in certain *rheopectic systems* which can be brought about by sound waves. The time of solidification of certain types of thixotropic systems is shortened by submitting them to a more or less regular movement, such as tapping the test tube containing the sol or slowly rolling it to and fro between the palms of the hands. This phenomenon is called *rheopexy* (27). Rheopectic solidification can be brought about by treatment with sound in those rheopectic systems which are more paste-like than gel-like, and whose particles are big enough to be influenced—by being either oriented or translocated—individually by the sound waves (82).

The last of the effects caused by the ponderomotive *action of sound waves* is their influence in *dilatant systems* (82). If the supernatant liquid is poured off the sediments of certain suspensions, such as quartz powder or sea sand, the sediments are somewhat moist and soft when only a slight pressure is applied. However, as soon as a stronger, one-sided pressure is applied, a sediment of this kind becomes dry and hard and offers considerable resistance to a penetrating instrument; as soon as the pressure ceases, the whole mass usually becomes moist again. This phenomenon is called *dilatancy* (64). In moist sand the particles tend to become very closely packed. If an external force displaces the particles, the packing becomes looser and consequently water is sucked in,

thus leaving the sediment apparently dry and hard. When a system normally exhibiting dilatancy is exposed to ultrasonics it is possible to drive an instrument through the sand without much resistance. It seems to hold true generally that dilatant and, therefore, non-plastic systems can be rendered somewhat plastic for the duration of intense sound treatment (82). This effect may become of some interest in drilling and molding operations.

All the described effects caused by intense sound treatment in liquid and solid systems are always accompanied by considerable *heat development* (30, 86). This thermal effect is particularly pronounced in the presence of extended phase boundaries, as they occur in microheterogeneous systems. Temperature increases in irradiated liquid samples of up to 1°C . in 3 sec. have been reported. Rises of 5 to 10°C . in temperature are common and are usually unavoidable when studying emulsification, etc.

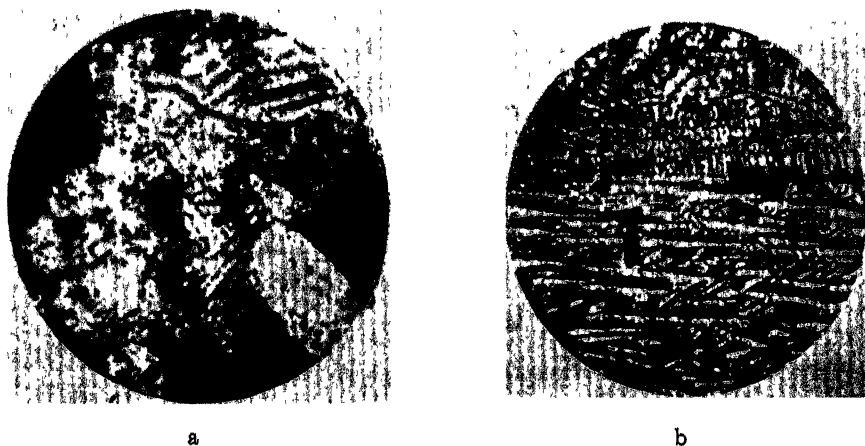


FIG. 12. The crystallization of zinc (a) without a sound field and (b) in a sound field. (From S. J. Sokoloff)

The heat effect is due to a number of factors, the relative importance of which depends on the system under consideration: absorption, gross stirring, the irregular reflection and the scattering of sound on interfaces, the friction between the particles and the oscillating dispersion medium, cavitation, etc. A detailed investigation of the heat effect in heterogeneous systems has never been carried out. The unavoidable heat development is a rather disturbing factor in many investigations, particularly in the range of ultrasonic frequencies. It has been suggested (29) that one might use it for medical purposes, such as the heating of joints, of the marrow inside intact bones, etc.

A discussion of the *chemical action of sound waves* is outside the framework of this review and can be referred to only briefly.

Schmitt, Johnson, and Olson (70) first showed that strong ultrasonic irradiation of water containing oxygen leads to the formation of hydrogen peroxide. This, as established later, is due to the activation of oxygen by the collapse of cavities.

In the presence of suitable reagents the activated oxygen, of course, may bring about many varied secondary oxidation reactions. The activation of oxygen is accompanied by the emission of light (sonoluminescence). The problem of sonic oxidation and sonoluminescence has been reviewed by Harvey (36).

Sound action may frequently change *labile states* to more stable ones. Such effects as the sudden boiling of superheated liquids or the detonation of highly sensitive explosives when subjected to sound waves (86) do not require further comment. Of interest here is the *spontaneous crystallization* from supersaturated solutions which frequently occurs (86) and which probably has considerable metallurgical importance (9, 24, 68, 69, 71); its mechanism is unknown. The crystalline structure of metals and alloys is changed by sonic treatment of their melts during solidification; the grain size is generally reduced and the growth of dendrites sometimes favored (figure 12). The results of different authors still vary widely, probably owing to differences in the systems investigated and the sound frequencies used.

A few general remarks must suffice for the *biological effects of sound*. In view of the disruptive and destructive, ponderomotive, and chemical actions of intense sound, it is small wonder that living systems are affected greatly by intense sound treatment. A very great number of observations have been reported. The reader is referred to the work of Harvey and collaborators (34, 35, 37), Chambers and collaborators (12, 14), and to the reviews of Dognon, Biancini, and Biancini (22) and Hiedemann (43).

SUMMARY

Ultrasonic (inaudible) and sonic (audible) sound waves alike bring about many phenomena of interest to the colloid chemist. They cause on the one hand all kinds of disruptive and destructive phenomena, such as emulsification, peptization, dispersion of certain solids in liquids, formation of fogs, etc.; on the other hand, they bring about orientation, accumulation, aggregation, and coagulation in liquid and gaseous colloidal and semi-colloidal systems.

The different methods of producing high-intensity sound waves are critically sketched, and the various mechanisms which cause these above-mentioned phenomena are reviewed.

Certain instances of possibly useful applications of sound waves are given. Sound waves offer a very convenient method for the study of emulsification and emulsions; emulsions may be produced or homogenized, and they may also be broken. Unstable suspensions may be coagulated. Sound waves further allow the colloidal dispersion of many metals in aqueous solutions, by the use of the Claus electrolytic method; also very fine grained photographic emulsions of great sensitivity can be produced. Immiscible metals may be dispersed in each other.

Sound waves may become a valuable tool in studying gels and systems showing anomalous viscosity.

Some proteins are denatured by sound waves; some are split to form seemingly well-defined fragments.

The coagulation of smokes and fogs by sound, though of an extremely complicated nature, seems to promise wide technical application.

Another useful application may come from the effect of sound waves on the structure of metals and alloys, when applied during their crystallization, and possibly also from the degassing action of sound on glasses and metallic melts.

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¹ An extensive review of the subject of sonic and ultrasonic waves in colloid chemistry by the author will be published in Volume V of Jerome Alexander's *Colloid Chemistry: Theoretical and Applied*, Reinhold Publishing Corporation, New York (in press).

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THE CHEMISTRY OF ISATIN

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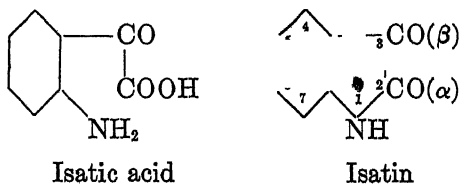
I. INTRODUCTION

In the course of studies of the action of various oxidizing agents on indigo, Erdmann (108, 109, 110) and Laurent (259, 260, 261, 262) in 1841 independently discovered an oxidation product which had the formula $C_8H_5NO_2$ and to which the name isatin was given. The preparation of the compound was best effected by the action of nitric acid or a mixture of nitric and chromic acids on indigo. When indigo was oxidized by chlorine, chloroisatin and dichloroisatin were obtained, while bromoisatin and dibromoisatin were prepared similarly through the agency of bromine. That the compound contained a benzene nucleus was shown by the fact that further action of chlorine on chloroisatin yielded chloranil.

In 1845 Hofmann (209) found that heating isatin with strong alkali gave aniline and that similar treatment of chloroisatin yielded chloroaniline. Subsequently Hofmann (210) also showed that treatment of isatin with nitrous acid gave 5-nitrosalicylic acid.

In 1866 and 1868 Baeyer (15, 24) published the results of his researches on the

reduction of isatin. Besides isatide, which had been obtained previously by Laurent (262, 265, 266) and by Erdmann (109), Baeyer obtained dioxindole, $C_8H_7NO_2$, and through further reduction of the latter compound oxindole, C_8H_7NO . Oxindole was further reduced to indole by passing its vapor over hot zinc oxide. Isatin was found to dissolve in alkali to give the salt of an acid, isatic (isatinic) acid. Kekulé (233) offered the suggestion that isatic acid was *o*-aminobenzoylformic acid and that isatin was its lactam, and thought that isatin might

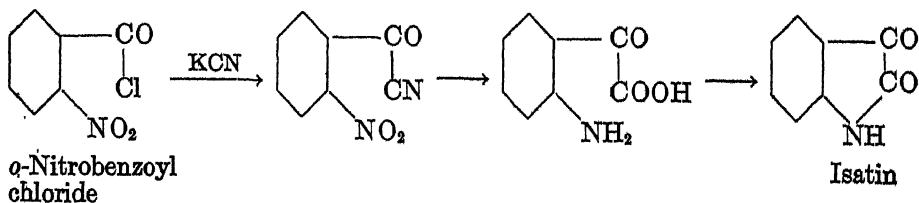


be synthesized from *o*-aminophenylacetic acid. Baeyer quickly realized that Kekulé's suggestion was correct and also saw the relationship of dioxindole and oxindole to isatin (16, 17).

Current practice in isatin nomenclature is to number the positions as shown in the formula above. The 2- and 3-positions are also frequently referred to as the α - and β -positions, respectively. Other systems of numbering have been used at times by some workers (170, 344) but the system shown here is used generally at the present time.

II. SYNTHESSES OF ISATIN AND OF ISATIN DERIVATIVES

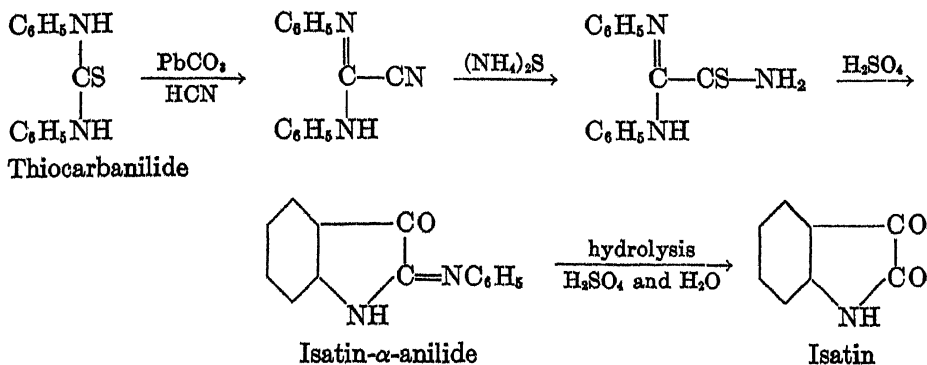
The synthesis of isatin from *o*-nitrobenzoyl chloride by Claisen and Shadwell (77) furnished definite confirmation of the structure suggested by Kekulé. The work of Kolbe (245) furnished further confirmation of its structure.



Isatin was also synthesized by Baeyer (20) and by Forrer (119) by the action of alkali on *o*-nitrophenylpropionic acid.

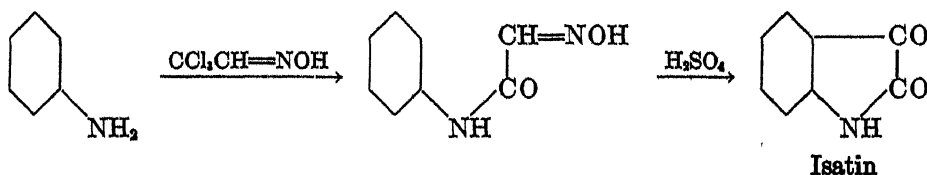
Baeyer (16) further synthesized oxindole by the reduction of *o*-nitrophenylacetic acid and later converted aminooxindole into isatin (17).

The synthesis of isatin and of many isatin derivatives has been accomplished through the utilization of two procedures developed by T. Sandmeyer. One of these methods starts with thiocarbanilide and is carried out according to the following scheme (39, 113, 347):



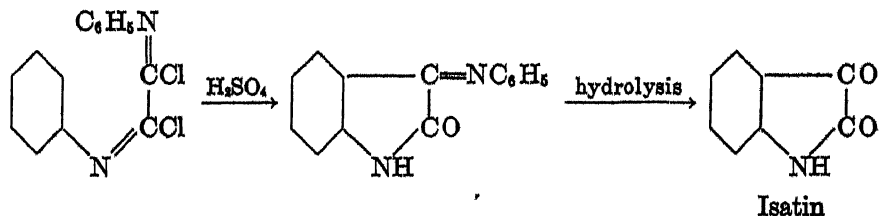
The isatin- α -anilide can be hydrolyzed quite readily to isatin.

The second of Sandmeyer's procedures (348) depends on the formation of isonitrosoacetanilide from aniline, chloral hydrate, and hydroxylamine. The isonitrosoacetanilide is converted into isatin on treatment with concentrated sulfuric acid.



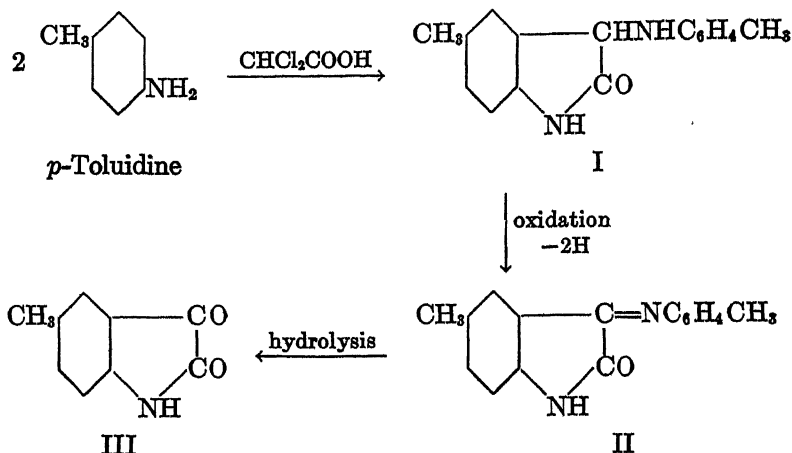
This method of synthesis has been used for the preparation of many isatin derivatives (49, 52, 90, 91, 144, 219, 290, 293, 294, 305, 338, 339, 364, 408). The nitroanilines form the corresponding nitroisonitrosoacetanilides, but these fail to give the nitroisatins when treated with sulfuric acid (46, 344).

Isatin and nuclear-substituted isatins have been prepared by Bauer (30, 31, 32) by the action of sulfuric acid on substituted imide chlorides of oxalic acid. This method has also been applied by Ostromisslensky (313), who has likewise

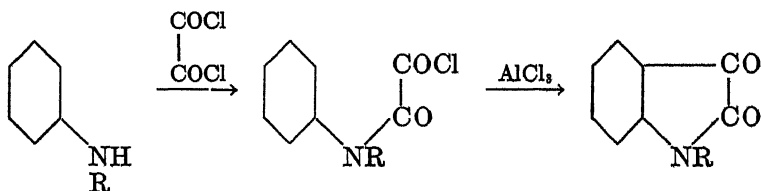


synthesized substituted isatins from *o*-toluidine and *p*-toluidine and dichloroacetic acid, in a reaction discovered by P. J. Meyer (300) and subsequently studied by Duisberg (101), Heller (170, 187), and Paucksch (314). The procedure depends on heating dichloroacetic acid with arylamines and results in the intermediate formation of an oxindole derivative (I). This derivative is then

oxidized to the corresponding isatin anil (II), which on hydrolysis gives the substituted isatin (III).

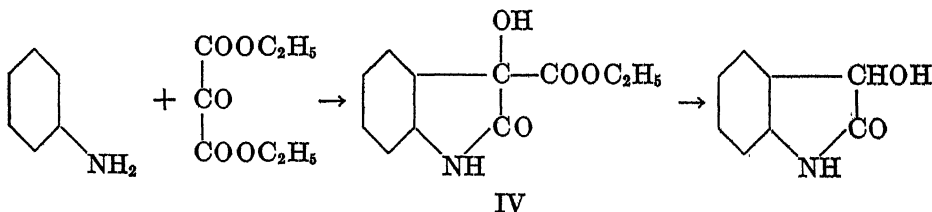


A method developed by Stollé (377, 378) provides a convenient procedure for the synthesis of many isatin derivatives (see also reference 253). In this synthesis an *N*-substituted aniline is treated with oxalyl chloride, and the resulting intermediate is converted into an isatin by treatment with anhydrous aluminum chloride.



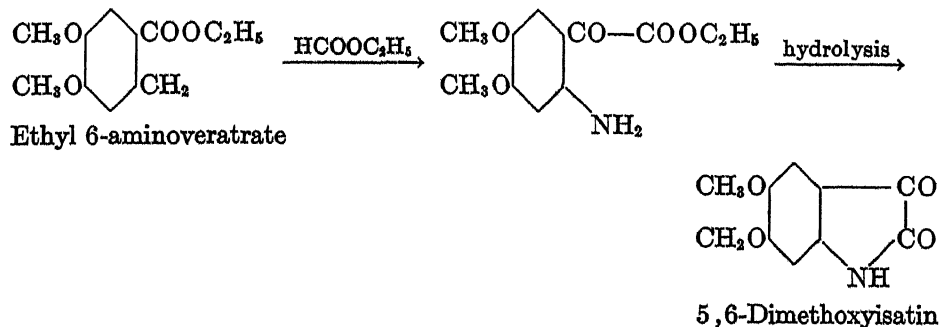
Cyanoformarylides also undergo similar ring closure on treatment with aluminum chloride or zinc chloride (53).

Another method for the synthesis of isatin derivatives is provided by a procedure developed by Martinet and coworkers (39, 146, 287, 288, 289, 292). Aniline or a substituted aromatic amine is condensed with the ethyl or methyl ester of oxomalonic acid. On treatment with alkali the resulting compound (IV) yields dioxindole (V) in the absence of oxygen. If the treatment with alkali takes place in the presence of air, isatin is obtained. This reaction has been

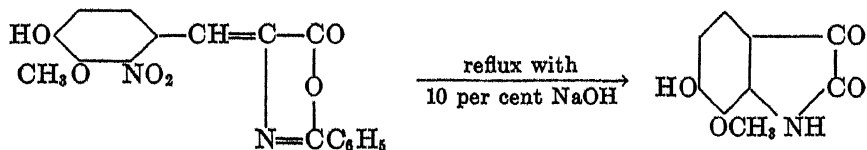


studied also by Kalb (229, 230) and by Halberkann (150). The method has been applied to the synthesis of α - and β -naphthisatins (207, 253, 287, 289).

Fetscher and Bogert (112) obtained 5,6-dimethoxyisatin from ethyl 6-amino-veratrate by means of the following series of reactions:

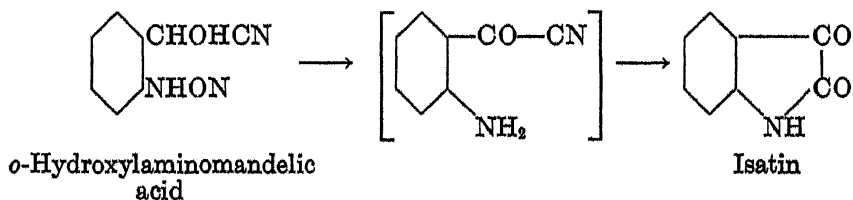


Burton and Stoves (66) prepared isatin derivatives by treating the azlactones of certain *o*-nitrobenzaldehydes with alkali.

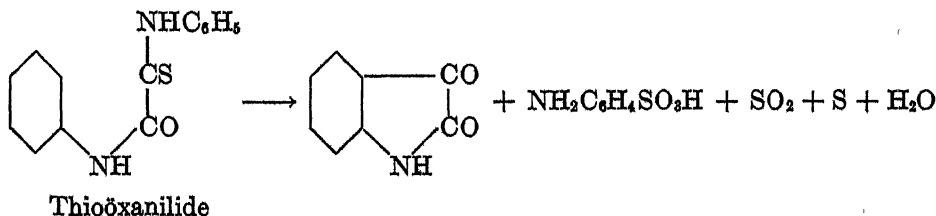


Succhara (383) obtained isatin-7-sulfonic acid by oxidizing quinoline-8-sulfonic acid with alkaline potassium permanganate.

Heller (168) obtained isatin by heating *o*-hydroxylaminomandelic acid with hydrochloric acid.



A synthesis developed by Reissert (332) depends on heating thiooxanilide with concentrated sulfuric acid.



Procedures have been developed by a considerable number of workers for preparing isatin and isatin derivatives by the oxidation of indigo or the appropriate indigo derivatives (93, 110, 119, 131, 201, 209, 230, 231, 235, 236, 250, 262, 326, 330, 391).

Böhm (38) has reported that isatin is formed in the body and excreted in the urine when *o*-nitrophenylglyoxylic acid is fed to rabbits. When *o*-nitromandelic acid is fed, dioxindole is found in the urine.

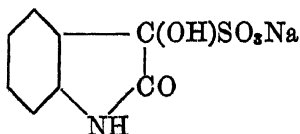
III. GENERAL PROPERTIES OF ISATIN

A. Physical properties

Isatin crystallizes from water, alcohol, or acetic acid in the form of red needles melting at 200–201°C. It is soluble in hot water, alcohol, acetic acid, and benzene but is sparingly soluble in ether. The substance is soluble in concentrated hydrochloric acid and in concentrated sulfuric acid. It dissolves in sodium or potassium hydroxide solution, forming the sodium or potassium salt of isatin. Heating the solution results in ring opening with the formation of the salt of isatic acid. Ring closure results when the solution is acidified, and isatin precipitates.

B. Salts of isatin

Laurent (264) and Schiff (349) found that isatin forms addition compounds with alkali bisulfites in a reaction typical of the β -carbonyl group.

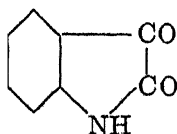
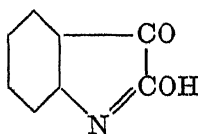


Isatin gives a stable perchlorate with perchloric acid (211). On the question of the constitution of the silver and alkali salts of isatin a lively controversy is found in the literature (76, 88, 153, 154, 169, 174, 176, 182, 183, 351). The sodium salt of isatin is obtained readily by the action of sodium ethylate on a solution of isatin in absolute alcohol. The potassium salt is prepared in similar fashion. The silver salt is prepared by adding the sodium salt of isatin to a solution of silver nitrate or by the action of silver acetate on a solution of isatin in alcohol.

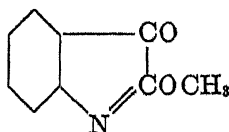
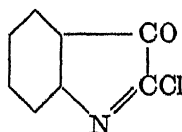
Peters (316) prepared the mercuric salt of isatin, while Hantzsch (157) prepared the mercurous salt. Of these metal salts the only ones of value in the synthesis of isatin derivatives are the silver salt (for *O*-alkyl derivatives) and the alkali salts (for *N*-derivatives).

C. Tautomerism

In 1882 Baeyer (22, 27) recognized the fact that isatin might be represented by either the lactam or the lactim structure.

Lactam structure
of isatinLactim structure
of isatin

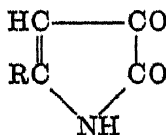
Toward many reagents isatin behaves as though it were the lactam, but *O*-alkyl ethers can be prepared from its silver salt and alkyl halides. Furthermore, isatin reacts with phosphorus pentachloride in hot benzene solution to give isatin α -chloride (18). It is said that isatin presents the first recognized case of a tau-

*O*-MethylisatinIsatin α -chloride

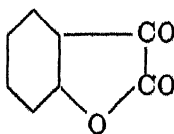
tomeric substance (366). Baeyer thought that isatin itself had the lactim form and termed the lactam "pseudoisatin." For a time it was thought that absorption-spectra measurements indicated that isatin was the lactam (86, 162, 247, 295, 306), but more recent work (7, 13, 80) leaves the question in doubt, since isatin and the lactam and lactim ethers have very similar absorption curves.

Claims have been made by G. Heller that numerous di- and tri-molecular isomers of isatin exist (174, 175, 176, 177, 178, 179, 181, 182, 183, 192, 194, 195, 196, 197). The claims of Heller have been disputed by Hantzsch and others (71, 153, 154, 155, 157, 158, 160). The details of this prolonged controversy can be found in the original papers and in part in Heller's own review (165).

Mumm and coworkers (307) have pointed out that 2,3-diketopyrrolines (I) are quite like isatin in appearance and properties and have suggested that these derivatives be regarded as mononuclear isatins. The oxygen analog of isatin



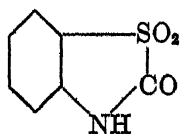
I



II

(II) has been studied by Stoermer (376) and by Fries (126) and coworkers.

Claasz (75) has prepared compound III, which he calls sulfurylisatin.

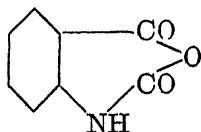


III

Sulfurylisatin

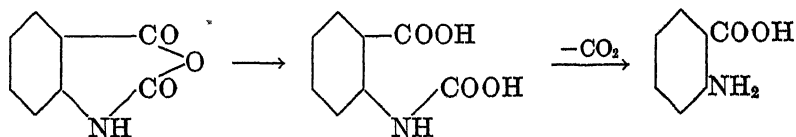
D. Oxidation and reduction

Kolbe (245) found that oxidation of isatin by chromic acid in acetic acid solution gave isatoic anhydride, a substance also obtained by Erdmann (107)



Isatoic anhydride

from anthranilic acid and phosgene (see also Friedlander and Wleugel (125)). Isatoic anhydride is converted into anthranilic acid by heating with mineral



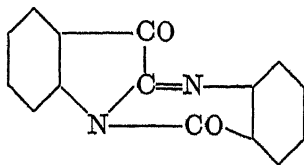
Isatoic anhydride

Anthranilic acid

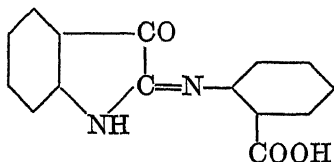
acids. On the other hand, *N*-acetylisatin is converted directly into *o*-acetamino-benzoic acid by oxidation with chromic acid. Nuclear-substituted isatins are converted into the corresponding isatoic acid anhydrides by chromic acid oxidation (36, 279, 315, 344).

Friedlander and Roschdestwensky (124) found that oxidation of isatin with potassium permanganate gave a compound designated as anhydroisatin- α -anthranilide, which was also obtained by Machemer (278) by the oxidation of indigo or isatin in pyridine by atmospheric oxygen in the presence of copper salts as catalysts.

The same compound is obtained by condensing isatin- α -anilide or isatin- α -chloride with anthranilic acid (95) or from the reaction of indoxyl and *o*-nitroso-benzoic acid.

Anhydroisatin- α -anthranilide

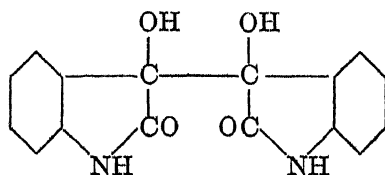
O-Methylisatin and anthranilic acid condense (189), giving isatin- α -anil-*o*-carboxylic acid



which on heating in acetic acid solution readily changes to anhydroisatin- α -anthranilide. Derivatives of anhydroisatin- α -anthranilide have also been prepared (195, 199).

Oxidation of isatin with alkaline hydrogen peroxide gives anthranilic acid, while substituted isatins similarly give the corresponding substituted anthranilic acids (230, 388, 391, 392).

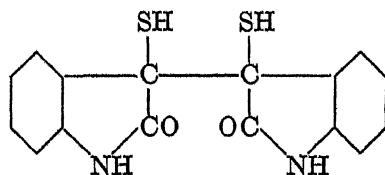
The first product obtained in the reduction of isatin is isatide (I) (19, 24, 110, 167, 259, 262, 265, 266, 399, 400, 401). This substance is also obtained by the



I

Isatide

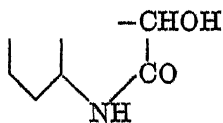
condensation of isatin and dioxindole in the presence of piperidine (152, 167, 399, 405). When isatin is reduced with hydrogen sulfide in alcoholic solution, the product is isatin thiopinacol (disulfisatide) (II) (260, 346, 397, 401, 402, 403, 404).



II

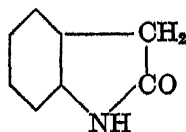
Isatin thiopinacol

Baeyer and Knop (15, 24) found that when isatin is reduced in acid solution the product is isatide. Further reduction by sodium amalgam gives 3-hydroxy-oxindole (dioxindole), which was also obtained by reducing isatin with sodium amalgam in alkaline medium. The further reduction of dioxindole by tin and



Dioxindole

mineral acids or by sodium amalgam and acid gave oxindole.

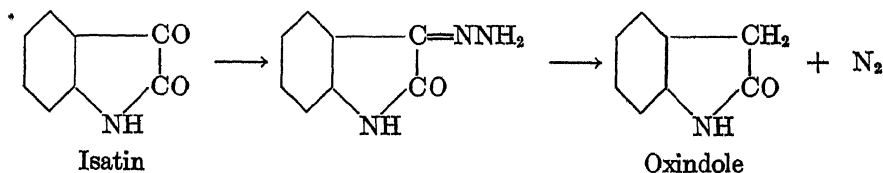


Oxindole

Isatin has also been reduced to dioxindole by Heller (167), using zinc and acetic acid, and by Marshalk (284, 285) and Kalb (229), using sodium hydrosulfite as the reducing agent. The procedure of Marshalk seems to offer the most satisfactory method for the preparation of dioxindole. Marshalk has further reduced dioxindole to oxindole through the action of sodium amalgam on an aqueous solution of dioxindole saturated with carbon dioxide.

The reduction of 1-methylisatin and of 1-ethylisatin by zinc and hydrochloric acid gives 1-methyldioxindole (78,390) and 1-ethyldioxindole (303), respectively. Sodium hydrosulfite can also be employed to convert other isatin derivatives to the corresponding dioxindoles (205, 390).

Curtius and Thun (84, 85) obtained oxindole from isatin through the agency of hydrazine. This reaction has also been studied by Borsche and Meyer (44)



and by Shapiro (363).

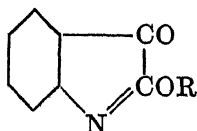
E. Acylation and alkylation

Suida (384) obtained *N*-acetyl isatin by heating isatin with acetic anhydride. The same derivative was described by Liebermann and Krauss (270) and was also prepared by Heller (177) from the sodium salt of isatin and acetyl chloride. *N*-Benzoyl isatin was prepared by Schwartz (361), Schotten (355), Liebermann and Krauss (270), and Heller (166, 169, 196). The compound is best prepared by the action of benzoyl chloride on the sodium salt of isatin or on a solution of isatin in pyridine. A number of other acyl derivatives of isatin and of substituted isatins have been described (299).

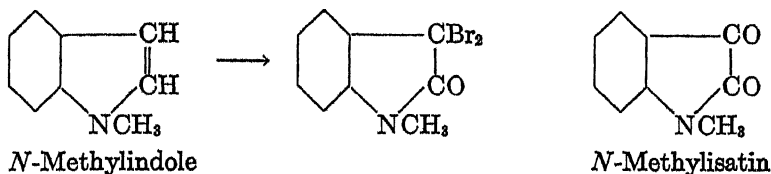
N-Benzenesulfonyl isatin has been prepared by Heller (178), while Aeschlimann (2) prepared *N*-phenylacetyl isatin by treating the sodium salt of isatin with phenylacetyl chloride.

The ethyl ester of isatin-*N*-carboxylic acid is obtained by treating the sodium salt of isatin with ethyl chloroformate (156, 177, 193, 196, 328). The sodium salt similarly reacts with ethyl chloroöxalate to give ethyl isatin-1-glyoxalate (177). Putochin (329) prepared ethyl isatin-1-acetate and diethyl isatin-1-malonate by treating the sodium salt of isatin with ethyl chloroacetate and ethyl chloromalonate, respectively. Isatin-1-acetic ester was also studied by Langenbeck (252) and by Ainley and Robinson (3).

Baeyer and Oekonomides (27) found that the silver salt of isatin reacted with alkyl halides to give *O*-alkyl derivatives of isatin (169).



On the other hand, the sodium and potassium salts of isatin react with alkyl halides and with alkyl sulfates to give *N*-alkyl derivatives (22, 28, 118, 169). *N*-Methylisatin was first prepared (78) from *N*-methyloxindole, which was converted by alkali hypobromite into 3,3-dibromo-*N*-methyloxindole. The latter compound was then hydrolyzed to *N*-methylisatin. This method is of importance because it establishes beyond doubt the position of the methyl

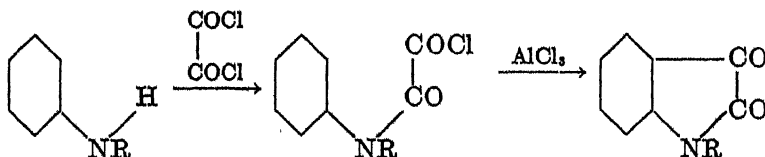


group.

The *O*-alkyl derivatives of isatin are easily hydrolyzed, while the *N*-alkyl derivatives are not. *O*-Methylisatin is stable when kept in a tightly stoppered bottle and protected from air and moisture; otherwise it changes rapidly to "methylisatoid".

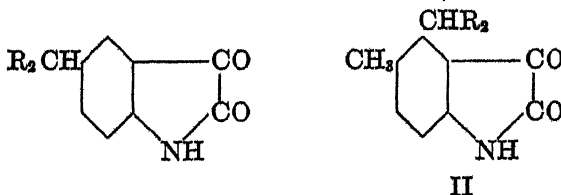
N-Methylisatin and *N*-ethylisatin can be prepared most conveniently by alkylating isatin by means of methyl sulfate and ethyl sulfate, respectively (43, 122, 242, 244).

The method of Stollé also provides a convenient preparation for the *N*-substituted isatins (378, 380, 381).



The metal salts of both *O*-methylisatin and *N*-methylisatin have been studied by Schlenk and Thal (351).

Reitzenstein and Breuning (337) have reported that tetramethyldiaminobenzohydrol condenses with isatin in the presence of concentrated sulfuric acid to give a compound of type I ($R = p(\text{CH}_3)_2\text{NC}_6\text{H}_4-$). In those cases where position 5 is occupied, the condensation is said to take place in position 4, yielding compounds of the type of II.



F. *N*-Hydroxyisatin

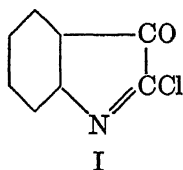
N-Hydroxyisatin and a number of its derivatives were prepared by Reissert (331, 333, 335), by Alessandri (5), and by Heller (168, 171, 200). The compound

is best prepared by the procedure developed by Arndt, Eistert, and Partale (11) through the action of benzoyl chloride on diazomethane. A product ($C_8H_5O_3N_3$) is obtained which gives *N*-hydroxyisatin when treated with dilute sulfuric acid. *N*-Hydroxyisatin is a red crystalline substance having the same melting point as isatin itself, 200–201°C. The compound was also obtained by Arndt, Eistert, and Partale (12) by treating *o*-nitrobenzoylcarbinol with sodium hydroxide.

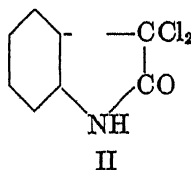
Unsuccessful attempts have been made by Neber and Keppler (312) and by Stollé (379) to prepare *N*-aminoisatin. Several derivatives of the compound have been described.

G. Halogenation, sulfonation, and nitration

Isatin reacts with phosphorus pentachloride in hot benzene solution to give isatin α -chloride (I) (18, 232). On the other hand, when isatin and phosphorus



I

Isatin α -chloride

II

3,3-Dichlorooxindole

pentachloride are allowed to react slowly at room temperature the product is 3,3-dichlorooxindole (II) (153). *N*-Alkyl derivatives of isatin react with phosphorus pentachloride to give the 1-alkyl-3,3-dichlorooxindoles (78, 118, 241, 244, 303, 378).

The direct chlorination of isatin gives 5-chloroisatin (89, 108, 109, 110, 209, 232, 259, 270, 280, 401) and 5,7-dichloroisatin (87, 89, 135, 136, 183, 228, 232). Chlorination of isatin or of 5-chloroisatin in aqueous suspension in the presence of potassium iodide or by addition of sodium hypochlorite to a suspension of isatin in 10 per cent hydrochloric acid gives 1,5-dichloroisatin (97). This substance when treated with sodium bisulfite and then acidified gives 5-chloroisatin, while heating with concentrated sulfuric acid converts it to 5,7-dichloroisatin. 5-Bromoisatin similarly gives 1-chloro-5-bromoisatin.

Bromination of isatin in alcohol in the cold gives 5-bromoisatin, while with the calculated quantity of bromine in hot solution 5,7-dibromoisatin is obtained (27, 89, 98, 134, 209, 219, 270, 274).

Isatin can be iodinated directly through the agency of iodine monochloride, the product being 5-iodoisatin (47, 230, 308). It has been claimed (357) that 5,7-diiodoisatin and a tetraiodoisatin can be obtained by the action of iodine monochloride on a solution of isatin in concentrated hydrochloric acid. It has been found, however, that such is not the case and that the sole product is 5-iodoisatin (391). It is apparently impossible to introduce an atom of iodine directly into the isatin nucleus in position 7 (388, 391).

5,7-Diiodoisatin and 5,6,7-triiodoisatin have both been prepared by indirect methods (230, 231, 391).

Isatin-5-sulfonic acid is obtained readily when isatin is treated with fuming sulfuric acid (291, 353).

The first nitration of isatin was effected by Baeyer (19) through the action of potassium nitrate on a solution of isatin in concentrated sulfuric acid. Baeyer reported that the compound melted at 226–230°C. and assumed that it was 5-nitroisatin. Liebermann and Krauss (270) employed essentially the same procedure and reported the melting point as 245°C. Rupe and Stocklin (345) reported that 6-nitroisatin was obtained when isatin was nitrated by the action of nitric acid in sulfuric acid solution. Calvery, Noller, and Adams (69) used a procedure essentially that of Rupe and Stocklin and obtained a product melting at 254–255°C. which they assumed to be Baeyer's 5-nitroisatin (compare also German patent 221,529). Rupe and Kersten (344) presented evidence purporting to show that Baeyer's product was 5-nitroisatin and that Rupe's nitroisatin was 6-nitroisatin.

The work of Baeyer, of Rupe, and of Calvery, Noller, and Adams was repeated by Sumpter and Jones (392), who found that the same product was obtained in each case and that it had been correctly characterized by Calvery, Noller, and Adams. The structure was established as 5-nitroisatin by oxidation in alkaline solution with hydrogen peroxide to 5-nitroanthranilic acid, which had been described previously by Bogert and Scatchard (37). 1-Methyl-5-nitroisatin was prepared by Borsche, Weussmann, and Fritzsche (46) by nitrating 1-methylisatin.

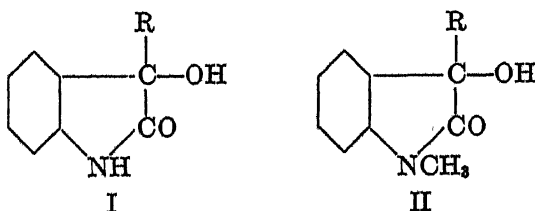
5-Nitroisatin was reduced catalytically to 5-aminodioxindole by Hartmann and Pannizzon (163). The acetyl derivative of the latter compound was oxidized to 5-acetaminoisatin, which on hydrolysis gave 5-aminoisatin. 5-Aminoisatin was converted to 5-hydroxyisatin by diazotization. It has also been reported (392a) that 5-nitroisatin can be reduced directly to 5-aminoisatin.

5,7-Dinitroisatin was prepared by Menon, Perkin, and Robinson (298) and by Guha and Basu-Mallik (142) through the action of potassium nitrate on isatin in sulfuric acid solution. The same compound is obtained when a solution of isatin in concentrated sulfuric acid is treated with two molecular proportions of nitric acid (390).

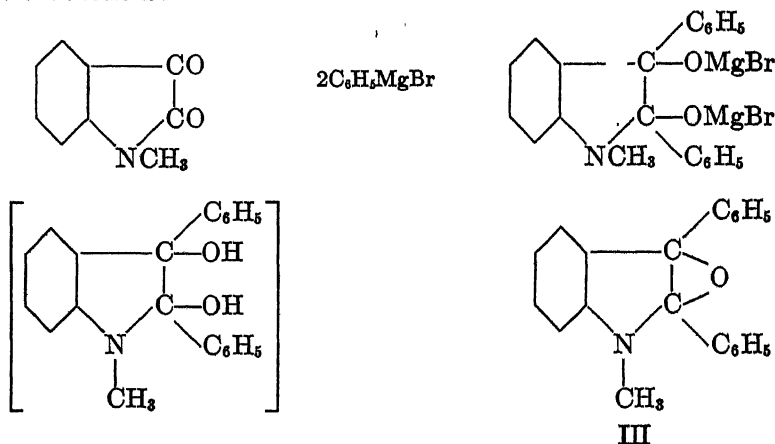
IV. REACTIONS OF ISATIN

A. Reaction with Grignard reagents

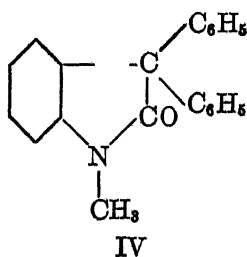
The reactions of isatin and its derivatives with Grignard reagents have been studied by Kohn and coworkers (238, 239, 242, 243, 244), by Myers and Lindwall (310), and by other workers (205, 369, 370, 373, 381, 387, 389). With the



Grignard reagents isatin yields the corresponding 3-alkyl(or aryl)-3-hydroxyoxindoles (I). Kohn (243) found that when phenylmagnesium bromide and *N*-methylisatin were allowed to react in equimolecular proportions the analogous 1-methyl-3-phenyl-3-hydroxyoxindole (II) was obtained. On the other hand, when *N*-methylisatin was treated with excess Grignard reagent Kohn found that both carbonyl groups in the isatin molecule reacted. He postulated the following scheme of reactions:

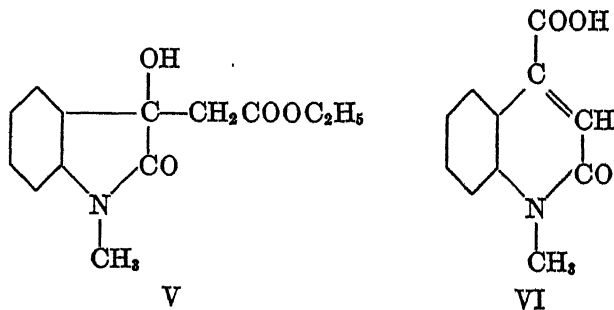


Myers and Lindwall (310) found that Kohn's compound III was not a single substance but a mixture of III with 3,3-diphenyl-1-methyloxindole (IV), formed from III by rearrangement. The reactions of *N*-substituted isatins with



3,3-Diphenyl-1-methyloxindole

Grignard reagents have also been studied by Stollé (381) and by Sumpter (387, 389).

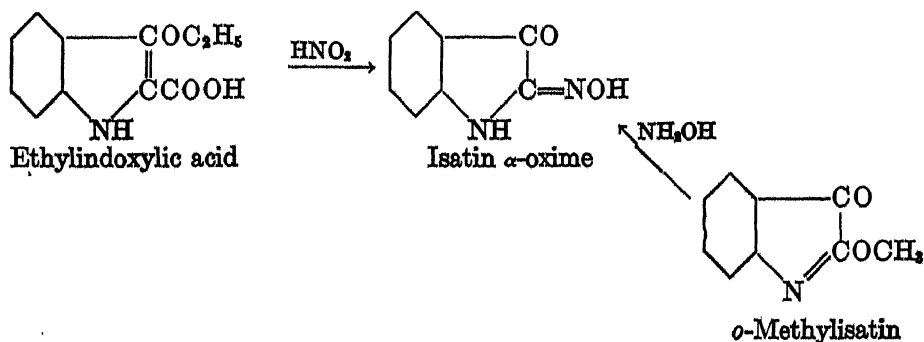


The application of the Reformatsky reaction in the isatin series was made by Myers and Lindwall (309). Isatin does not react in the sense of the Reformatsky reaction, but the *N*-substituted isatins react with zinc and ethyl bromoacetate to give the ethyl ester of (3-hydroxy-1-alkyloxindolyl-3-)acetic acid (V). Hydrolysis of this ester brings about ring opening and subsequent closure to give the quinoline derivative (VI) (compare Aeschlimann (2)).

B. Reaction with hydroxylamine, phenylhydrazine, and aromatic amines

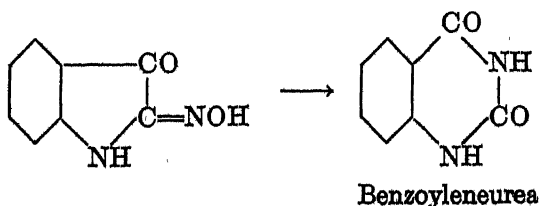
Isatin reacts with hydroxylamine to give isatin β -oxime (23, 45, 59, 129, 130, 159, 243, 249, 280, 414). Isatin β -oxime is identical with nitrosooxindole and can be prepared by treating oxindole with nitrous acid (24, 46, 129, 148, 149, 243). Direct reaction of isatin and hydroxylamine gives only the β -oxime (249). Isatin dioxime has been prepared from isatin- α -imine and hydroxylamine (335). It is quite readily hydrolyzed and gives isatin β -oxime on heating with aqueous hydrochloric acid. Both carbonyl groups in acetylisatin react directly with hydroxylamine to give the dioxime (249, 359).

Isatin α -oxime was prepared by Baeyer (21, 22) by the action of nitrous acid on ethylindoxylic acid. Heller (174) obtained the same compound by treating *O*-methylisatin with hydroxylamine.

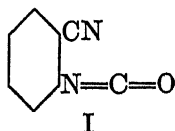


The preparation of the isatin oximes has also been studied by Wieland and coworkers (409, 410). The reduction of both the α - and the β -oximes by zinc dust and acetic acid, followed by treatment with ferrous chloride or nitrous acid, gives isatin.

On standing in alkaline solution both isatin α -oxime and its ethyl ether are converted into benzoyleneurea through a Beckmann rearrangement (174). The

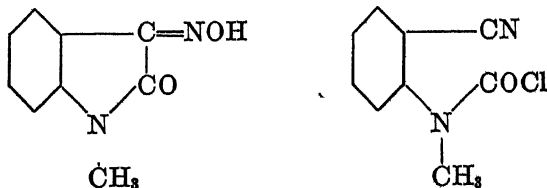


β -oxime does not undergo this rearrangement but with phosphorus pentachloride it gives *o*-cyanophenyl isocyanate (I) (45).



I
o-Cyanophenyl isocyanate

N-Methyl- β -isatoxime similarly gives the chloride of *N*-methyl-*o*-cyanophenyl-carbamic acid. Beckmann and Bark (34) obtained *o*-cyanophenyl isocyanate

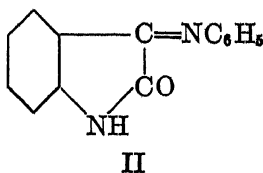


N-Methyl- β -isatoxime

by the action of phosphorus pentachloride and phosphorus oxychloride on *N*-acetylisonitrosoacetophenone. Heating isatin β -oxime to 230°C. results in decomposition, yielding *o*-aminobenzonitrile (46). Isatin β -oxime forms insoluble salts with a number of metal ions. Its use as an analytical reagent has been discussed by Hovorka and Šfkora (212, 213).

Isatin reacts with phenylhydrazine to give isatin β -phenylhydrazone (22, 116, 251, 341). The α -phenylhydrazones are prepared by treating the corresponding isatin-*O*-ether or isatin α -chloride with phenylhydrazine (4, 14, 172, 336, 359). Heller has prepared isatin diphenylhydrazone (171) from isatin α -phenylhydrazone and phenylhydrazine. Panaotovic (315) has obtained diphenylhydrazones (osazones) from acetylisonitrosoacetophenone and its derivatives. Reissert and Hessert (335) have described isatin α -semicarbazone, while the β -semicarbazone was prepared by Marchlewski (281).

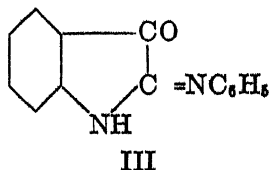
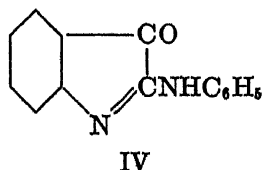
Isatin reacts directly with aniline in alcoholic solution to give isatin- β -anilide (II) (36, 106, 236, 304, 313, 325, 327, 349, 380, 396). Isatin- α -anilide (III) was



II
Isatin- β -anilide

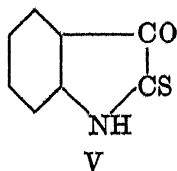
obtained by Sandmeyer in his synthesis of isatin from thiocarbanilide (347). The compound was prepared also by Pummerer and Götter from nitrosobenzene and indoxyllic acid (92, 325, 327). According to Pummerer the compound exists

in two forms (III and IV), the tautomerism having been confirmed by Callow and Hope (68) (compare also references 111 and 343).

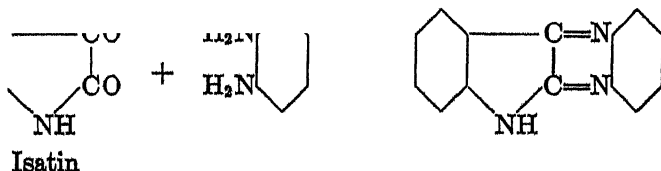
Isatin- α -anilideIsatin- α -anilide

Heating isatin- α -anilide with aniline gives isatindianilide (169), which can also be prepared from *O*-methylisatin and aniline. Isatin- α -anilide and phenylhydrazine react to give isatin α -phenylhydrazone (169, 336).

Isatin- α -anilide reacts with hydrogen sulfide in acid solution to give α -thioisatin (V) (347).

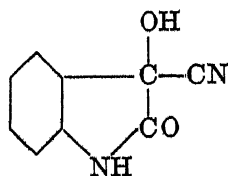
 α -Thioisatin

Isatin condenses with *o*-phenylenediamine as shown in the scheme below (61, 247, 281, 283, 360):

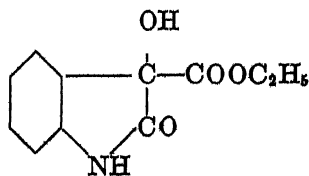


C. Reaction with hydrocyanic acid, ammonia, and aliphatic amines

Heller (198) found that isatin reacts with hydrocyanic acid to give a compound ($C_9H_6O_2N_2$) which he called hydrocyanisatin. Kalb (229) showed that the compound possessed the structure I by converting it into ethyl dioxindole-3-

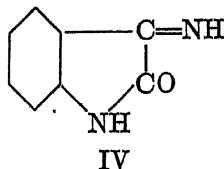
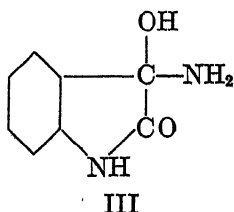


Hydrocyanisatin

Ethyl
dioxindole-3-carboxylate

carboxylate (II) by the action of alcoholic hydrochloric acid. The latter ester was synthesized also from aniline and oxomalonate ester. Martinet (248) found that 1,7-trimethyleneisatin reacted with hydrocyanic acid in the same manner, while the 5,7-diiodo derivative of I was prepared by Kalb and Barrer (230).

Reissert and Hoppmann (336) found that treatment of a cold suspension of isatin in alcohol with dry ammonia results in the separation of colorless crystals of compound III, which decomposes in air to isatin and ammonia. If the treat-

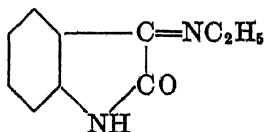


Isatin- β -imide

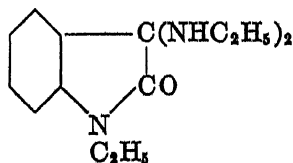
ment with ammonia is interrupted before crystals appear and the mixture is allowed to stand, isatin- β -imide (IV) separates. This reaction has been studied also by Laurent (263), Sommaruga (367, 368), and Jacini (224). Isatin- β -imide gives isatin- β -anilide on treatment with aniline.

Isatin- α -imide has been prepared by Reissert and Hessert (335) from an intermediate product obtained in the reduction of *o*-nitromandelonitrile.

Haslinger (164) found that isatin reacts with alcoholic ethylamine to give isatin- β -ethylimide (V); he also reported the formation of 1-ethyl-3,3-bis-(ethylamino)oxindole (VI) when an excess of diethylamine is used. Just how



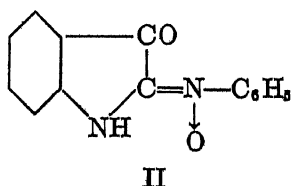
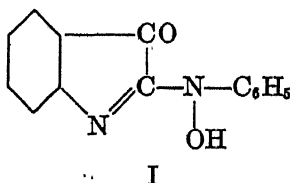
Isatin- β -ethylimide



N-alkylation could take place in this reaction remains obscure.

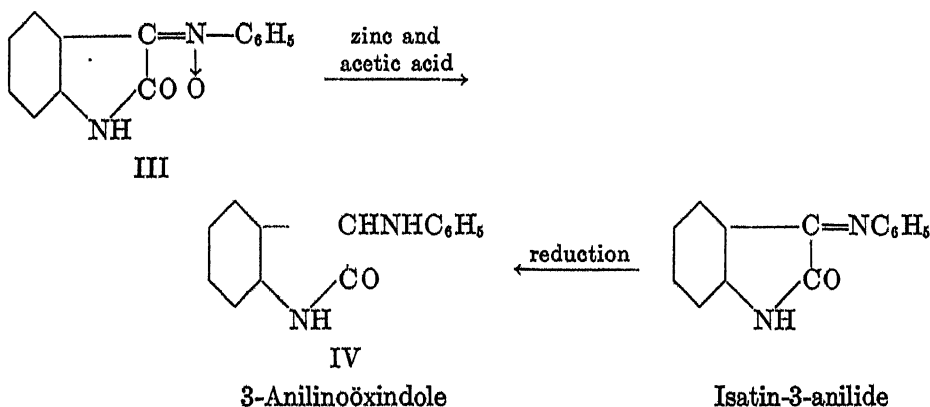
D. Reaction with phenylhydroxylamine

Isatin condenses with β -phenylhydroxylamine to give a product which Rupe formulated as the α -derivative (lactim form) (I). Rupe (342, 343, 344, 345)



concluded that the product was an α -derivative of isatin from the fact that it was formed also from isatin- α -chloride and β -phenylhydroxylamine. It appears that evidence drawn from such a reaction is not always trustworthy, since Naimen and Bogert (311) found that both α - and β -derivatives of isatin can be obtained from isatin- α -chloride, depending on experimental conditions. Heller (184) suggested that Rupe's compound was the β -derivative (III).

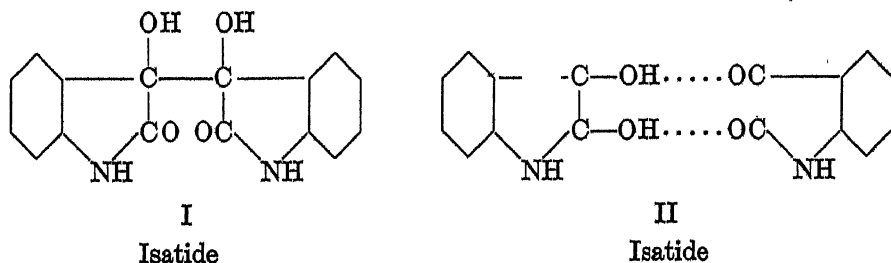
Alessandri (5) prepared from *o*-nitrophenylacetylene and nitrobenzene a compound which he formulated as (II) the lactam of I. Alessandri's compound on mild reduction gave isatin- α -anilide and with phenylhydrazine gave isatin- α -phenylhydrazone. On the other hand Rupe's compound gave isatin β -phenylhydrazone (345) with phenylhydrazine and on reduction gave 3-anilinoöxindole (IV), identical with the compound obtained by reducing isatin-3-anilide (68).



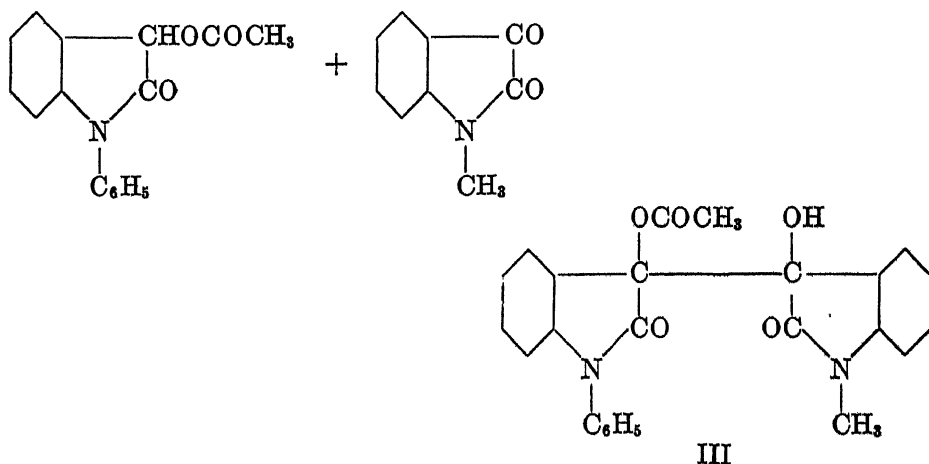
The evidence seems conclusive that Rupe's product is the β -derivative (III) and not the α -derivative (I or II), as maintained by Rupe.

E. Reaction with dioxindole, oxindole, and indoxyl

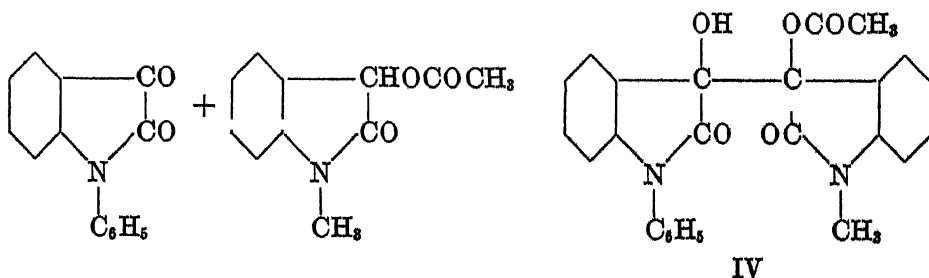
Isatin condenses readily with dioxindole in the presence of piperidine to give isatide. This same very insoluble substance is very frequently encountered in the reduction of isatin. The most generally accepted structure for isatide is that of isatin pinacol (I) (152, 240, 241, 242, 244, 268, 382, 385, 403). On the other hand Heller has proposed a quinhydrone structure (II) for isatide (174,



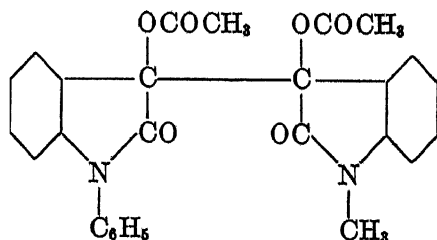
186, 197). The problem of the constitution of isatide seems to have been resolved in favor of the isatin pinacol formula by the work of Stollé and Merkle (382). These workers found that 1-alkyl(or aryl)-3-acyldioxindoles were capable of condensing with isatins to give the corresponding acyl isatides. Since the 3-acyldioxindoles condense quite as readily as do the dioxindoles having a free



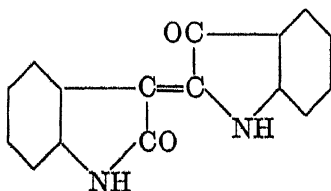
hydroxyl group in position 3, the formulation of Heller is untenable. In further support of the pinacol formula Stollé found that compound III and the isomer IV obtained from *N*-phenylisatin and 1-methyl-3-acetyldioxindole gave the same



diacetyl derivative (V) when treated with acetic anhydride.



Isatin condenses with indoxyl to give indirubin (VI) (51, 111, 123, 312, 398,

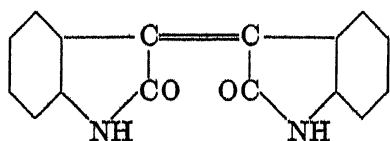


VI

Indirubin

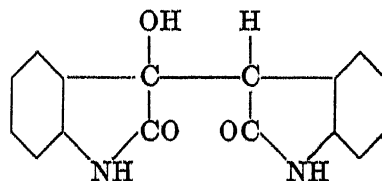
400), which can also be prepared from isatin- α -chloride and oxindole (398).

Isatin and oxindole condense in acid media to give isoindigo (VII) (114, 152, 259, 277, 312, 398, 399, 400, 401, 403) and in the presence of pyridine to give isatane (VIII) (152, 261, 268, 277, 399, 403).



VII

Isoindigo

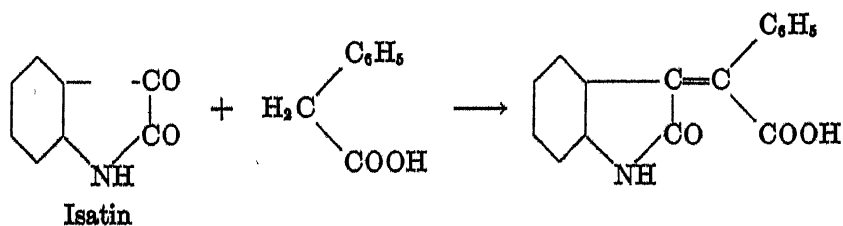


VIII

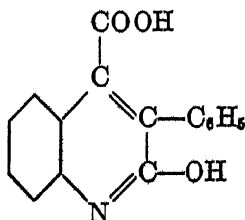
Isatane

F. Condensation with reactive methylene groups

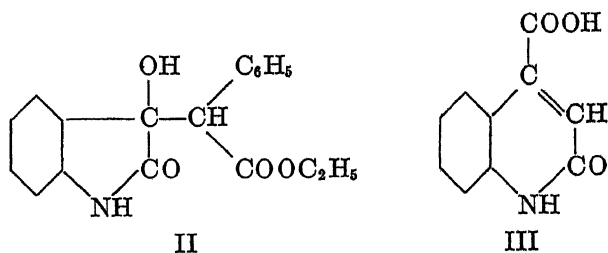
Isatin was condensed with phenylacetic acid by Gysae (147), who formulated the reaction as shown below. It was found later by Borsche and Jacobs (43)



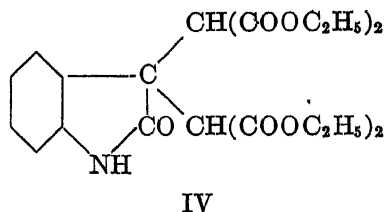
that the product was α -hydroxy- β -phenylcinchoninic acid (I), identical with the product obtained by Hübner (214) from isatin and phenylacetic anhydride.



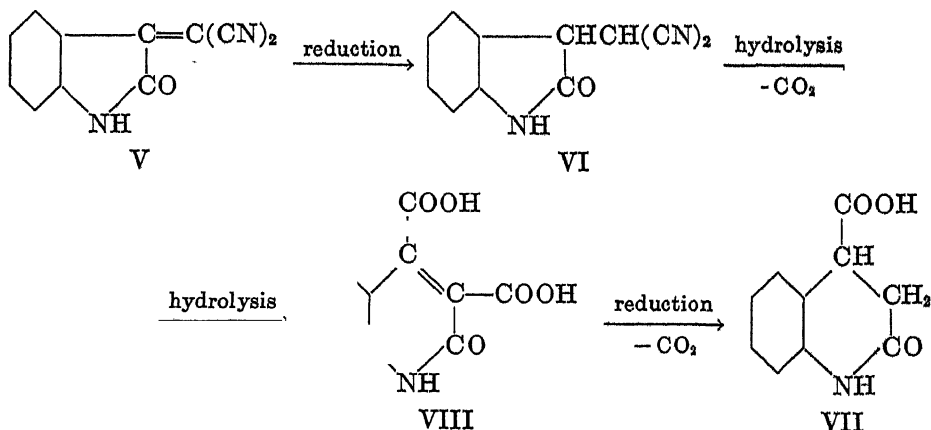
Zrike and Lindwall (416) condensed ethyl phenylacetate with isatin, obtaining a product (II) which on hydrolysis gave compound I. Condensation of isatin



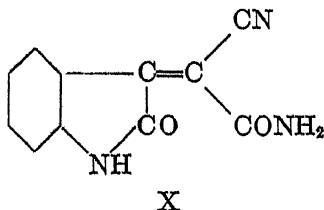
with malonic acid gives α -hydroxycinchoninic acid (III, written here as the lactam) (2, 43). The same compound is obtained when 1-acetylisatin is heated with alkali (2, 70, 315). Isatin condenses with diethyl malonate in the presence of diethylamine to give the tetraethyl ester of 3,3-bis(methane-diacid)oxindole (IV) (276).



Isatin and malonitrile were condensed by Walter (405), the product being compound V. The latter compound was also prepared by Zrike and Lindwall (416) and reduced to VI, which on hydrolysis gave 1,2,3,4-tetrahydro-2-quinolone-4-acid (VII). Hydrolysis of compound V by concentrated hydrochloric acid gave a dibasic acid, $C_{11}H_7O_5N$, which Zrike and Lindwall regarded as probably having the structure VIII (and which lost carbon dioxide on reduction, giving VII). This dibasic acid did not melt below $340^\circ C$., but treatment of its disilver salt with ethyl iodide gave the diethyl ester (IX), m.p. $150-151^\circ C$. The same dibasic acid (VIII) was also obtained by the hydrolysis of IV and of the

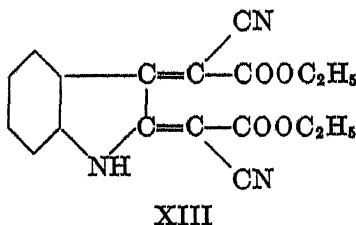
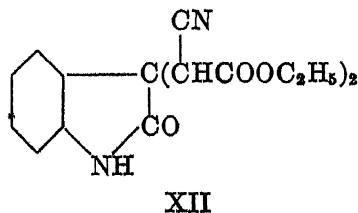
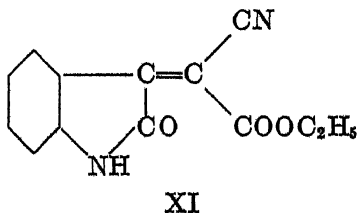


product (X) obtained by condensing isatin with cyanacetamide (416). Isatin also condenses with malonamide and with malonanilide mole for mole to give the



corresponding isoindigoid derivatives.

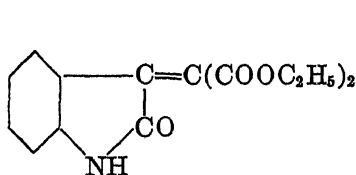
The condensation of isatin with ethyl cyanoacetate has been studied by Hill and Samachson (204), Yokayama (413), and Sumpter (390). The reaction takes place in absolute alcohol in the presence of diethylamine or piperidine to give a product, $C_{18}H_{17}O_5N_3$, which melts at about 116°C . (depending on the rate of heating) with decomposition into ethyl cyanoacetate and compound XI. The condensation product was formulated by Hill and Samachson as XII, while Yokayama thought it to be the hydrate of XIII. There appears to be no analogy in isatin chemistry for the formation of XIII, which should be highly colored. The lack of color and general properties of the compound suggest that formula XII is correct.



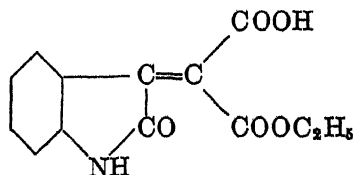
Hill and Samachson found that hydrolysis of XI by concentrated hydrochloric acid gave a product (not analytically pure) melting above 300°C ., which on reduction gave VII. More recent work (390) has shown that this hydrolytic product is identical with compound VIII of Zrike and Lindwall, since it is readily converted into the diethyl ester (IX).

Yokayama reported that treatment of XI with concentrated sulfuric acid and absolute alcohol gave XIV (m.p. 149°C .) and XV (m.p. 219°C .). Treatment of XV with sodium hydroxide gave VIII (Yokayama incorrectly reported the melt-

ing point 304–305°C.). Yokayama's compound XIV (m.p. 149°C.) has been found to be identical with compound IX (390).



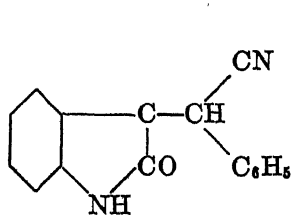
XIV



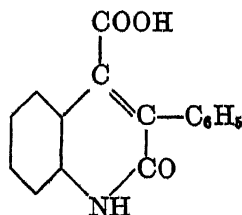
XV

Compounds having structures XIV and XV should be highly colored, while the actual products are colorless and are better formulated as the diethyl ester (IX) and the monoethyl ester of VIII, respectively.

Analogous results were obtained by Hill and Samachson in condensing 5-iodoisatin and 5,7-dibromoisatin with ethyl cyanoacetate. These same investigators also found that isatin and phenylacetonitrile condense to give 3-(phenylcyanomethyl)-3-hydroxyoxindole (XVI), which on hydrolysis gives 2-quinolone-3-phenyl-4-carboxylic acid (XVII).

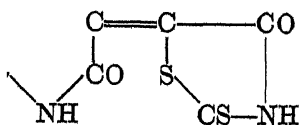


XVI

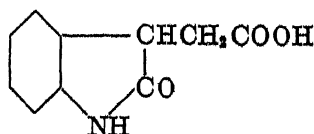


XVII

Gränacher and Mahal (133) obtained β -rhodanal oxindole (XVIII) by condensing isatin and rhodanine. β -Rhodanal oxindole on reduction and hydrolysis



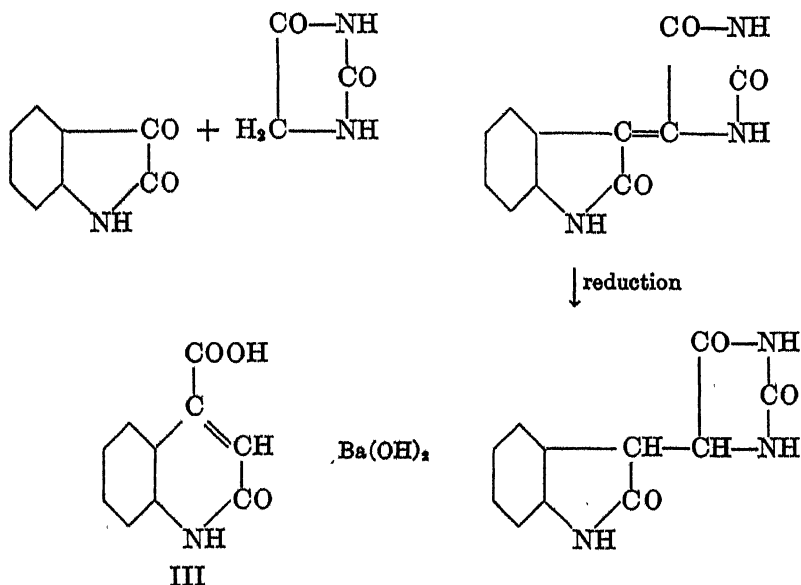
XVIII



XIX

gave a compound which Gränacher described as oxindoleacetic acid (XIX), but which was shown by Hill, Schultz, and Lindwall (206) to be 2-keto-1,2,3,4-tetrahydroquinoline-4-carboxylic acid (VII). The synthesis of rhodanine oxindoles has been studied also by Andreasch (6) and by R. V. Jones and Henze (227), while the condensation of rhodanic acids with isatin and with nitroisatin has been studied by Hann (151) and by H. A. Jones and Hann (226).

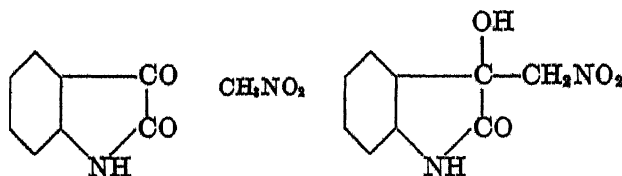
The condensation of isatin with hydantoin has been studied by Hill and co-workers (203, 206). The results obtained by these investigators may be summarized in the following scheme:



Reduction of compound III gave compound VII, which was identical with Gränacher's "oxindole acetic acid". Furthermore, compound III was found to be identical with the product obtained by Camps (70) from acetylisatin and sodium hydroxide and by Borsche and Jacobs (43) from malonic acid and isatin.

The reaction of isatin and hydantoin was also studied by Kotake (248), whose results were not in complete accord with those of Hill, Schultz, and Lindwall (206). The researches were repeated by Henze and Blair (202), who found the work of Hill, Schultz, and Lindwall correct.

Isatin and substituted isatins condense with nitromethane and nitroethane to give aldol-like condensation products (79, 81, 416).

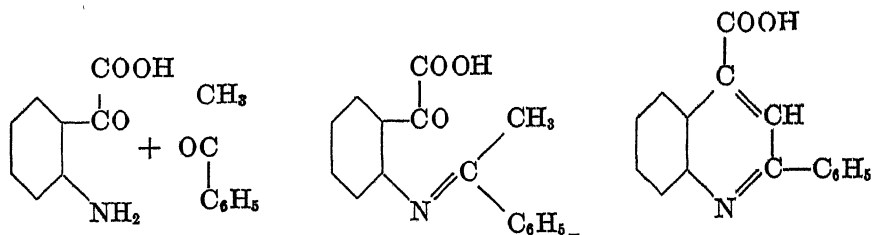


G. Condensation with ketones

Pfützing (317, 318, 319, 320) obtained substituted cinchoninic acids when isatin was condensed with methyl ketones in the presence of concentrated potassium hydroxide, the reaction mechanism perhaps being that indicated. Compound I (p. 418) is the drug cinchophen (atophen).

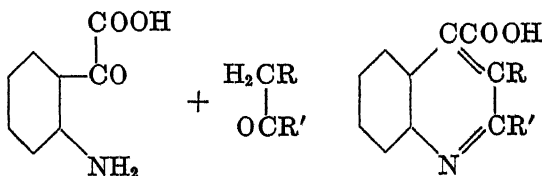
Ethyl methyl ketone condenses with isatin in the Pfützing reaction to give both 2,3-dimethylcinchoninic acid (319) and 2-ethylcinchoninic acid (50). The Pfützing reaction has been studied by Lindwall and coworkers (48, 102, 103, 274, 275, 416), Henze and coworkers (67, 83, 269), Crippa and Scevola (82), v.

Braun and coworkers (49, 50, 51), and Steinkopf (371), and its applications have been extended to the synthesis of a large number of cinchoninic acids.

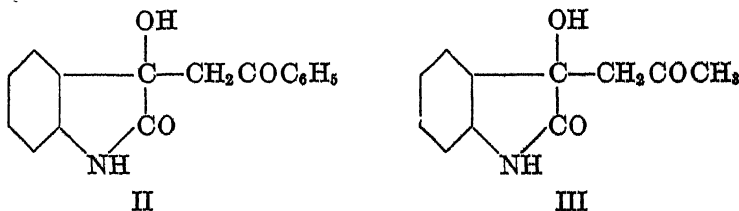


Cinchophen

Isatic acid was condensed by v. Walther (406) with iminonitriles, $\text{NH}=\text{CRCH}_2\text{CN}$, to give 2-alkyl-3-cyanocinchoninic acids (406). The reaction was further utilized by Pfitzinger (320) for the synthesis of 2,3-dialkylcinchoninic acids.



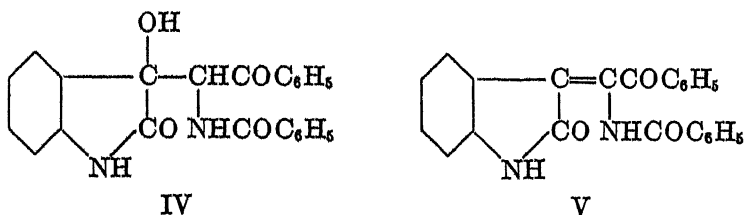
Lindwall and coworkers (48, 102, 103, 275, 416) found that acetophenone condenses with isatin under the influence of mildly basic condensing agents to give 3-hydroxy-3-phenacyloxindole (II).



Similarly, acetone condenses with isatin in the presence of diethylamine to give 3-acetonyl-3-hydroxyoxindole (III), and in the presence of concentrated alcoholic potassium hydroxide to give 2-methylcinchoninic acid. 6,8-Dibromo-2-methylcinchoninic acid was prepared similarly from 5,7-dibromoisatin by Lawson, Perkin, and Robinson (267). The aldol-like products, II and III, are readily dehydrated to yield 3-phenacylideneoxindole and 3-acetonylideneoxindole, respectively. Refluxing 3-phenacylideneoxindole with mineral acids causes rearrangement to cinchophen (I) (102, 416). John found that the condensation of benzalacetone with isatin gave 2-styrylquinoline-4-carboxylic acid. Benzylacetone condensed with isatin under the conditions of the Pfitzinger reaction gives 2-phenylethylquinoline-4-carboxylic acid (82).

At room temperature isatin condenses with benzoylphenacylamine (103) in

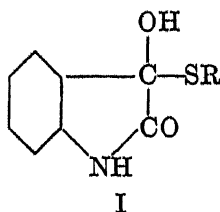
the presence of piperidine to give 3-hydroxy-3-(α -benzoylamino)phenacyloxindole (IV), while at higher temperature the product is 3-(α -benzoylamino)phenacyloxindole (V). Reduction of V and subsequent hydrolysis of the reduction



product gave cinchophen (I).

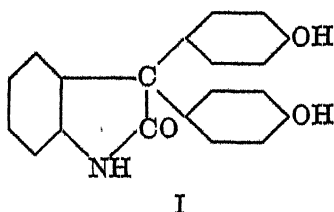
H. Reaction with mercaptans

Baumann (33) and Schönberg, Schütz, Arend, and Peter (354) found that isatin and acetylisatin react with mercaptans to give 1:1 addition products probably having the structure shown below (I):



I. Condensation with phenol, toluene, and tertiary amines

Baeyer and Lazarus (25, 26) and subsequently Liebermann and Danaila (272) found that isatin condenses with toluene, phenol, resorcinol, anisole, α -naphthol, and aromatic secondary and tertiary amines to give oxindole derivatives of the type of phenolisatin (3,3-bis(4'-hydroxyphenyl)oxindole) (I). In general it was



Phenolisatin

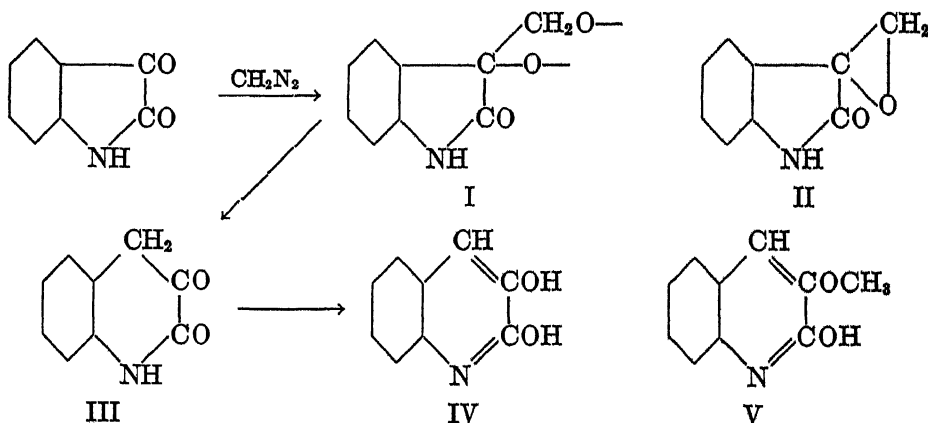
assumed, in agreement with Baeyer, that these condensation products were 3,3-derivatives of oxindole (on the contrary compare Sen (362)), but it remained for Inagaki to establish definitely their structure (216, 217, 218, 219, 220, 221). The condensation of α -naphthol with isatin has been studied by Candea (71), Steopoe (375), and Gabel and Zubarovskii (127).

Phenolisatin (I) and its diacetyl derivative, isacene, have found use pharmacologically as mild purgatives (35, 56, 73, 98, 365, 407).

A number of condensation products of *N*-benzylisatinsulfonic acid with various phenols are said to be of value as mothproofing agents (56).

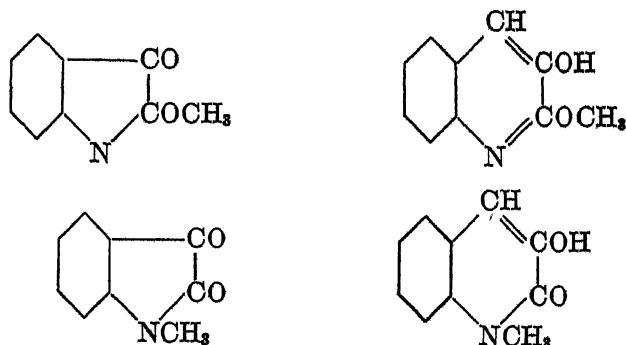
J. Reaction with diazomethane and hydrazoic acid

The reaction of isatin with diazomethane was first studied by Heller (180, 191), who found the principal product to be 2,3-dihydroxyquinoline (III or IV). In his second paper on this subject Heller reported obtaining a second product, possibly the isoquinoline derivative. The reaction was later studied by Arndt and coworkers (9, 10). Arndt assumed that the diazomethane reacts with the β -carbonyl group of isatin to give an addition product (I), which then rearranges to give either the ethylene oxide (II) or 2,3-dihydroxyquinoline (III or IV). Arndt reports increasing yields of V as the reaction period is increased. The



reactions of a number of substituted isatins with diazomethane have been studied by Heller (191). The reaction of 1-hydroxyisatin with diazomethane was also investigated by Arndt (9).

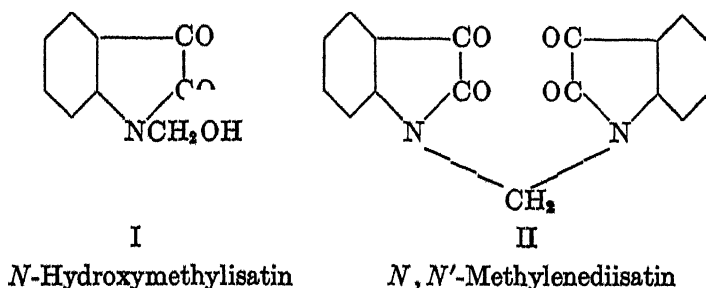
This reaction has likewise been investigated by Ault, Hirst, and Morton (13), who prepared quinoline derivatives from isatin, *O*-methylisatin, and *N*-methylisatin.



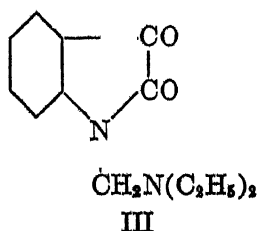
Caronna (72) found that isatin and *N*-acetylisatin react with hydrazoic acid to give anthranilamide, while *N*-ethylisatin reacts similarly to give *o*-ethylamino-benzamide.

K. Reaction with formaldehyde and phenyl isocyanate

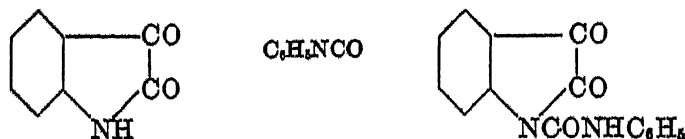
Reissert and Händeler (334) found that isatin and formaldehyde react to give *N*-hydroxymethylisatin (I) and *N,N'*-methylenediisatin (II).



Einhorn and Göttler (105) treated isatin with formaldehyde and secondary amines, obtaining thereby derivatives of the type of *N*-diethylaminomethyl isatin (III).



Gumpert (145) and Goldschmidt and Meissler (132) found that the imino hydrogen atom of isatin reacts with phenyl isocyanate in the usual manner.



L. Table of condensation reactions

A complete listing of the formation of all of the indigoid dyes and other condensation products which have been prepared from isatin and isatin derivatives would require many pages. A comprehensive listing of such reactions has been given by Heller in his review (165). In view of this fact no attempt is made to repeat that listing here. In table 1 will be found a listing of the references to those condensation reactions of isatin and derivatives which have been studied since the publication of Heller's review and which are not discussed elsewhere in this article.

TABLE 1
Condensation reactions of isatin and isatin derivatives

SUBSTANCE REACTING WITH ISATIN OR AN ISATIN DERIVATIVE	
Acetophenone.....	(415)
Aniline.....	(54)
<i>o</i> -Anisidine.....	(54)
Amylresol.....	(99, 100)
Amylphenol.....	(99)
2-Benzylbenzimidazole.....	(395)
<i>o</i> -Benzylphenol.....	(98)
<i>o,o'</i> -Biphenol.....	(98)
Bromoacetophenone.....	(274)
3-Chloro-2-hydroxybiphenyl.....	(394)
Cryptopyrrole.....	(322)
Cryptopyrrolidine.....	(138)
2,3-Diaminoquinoxaline.....	(104)
Dihydroisindole.....	(138)
Diketopiperazine.....	(206)
Dimethylaniline.....	(381)
Dimethylalkoxythionaphtheneones.....	(120)
2,4-Dinitrophenylhydrazine.....	(340)
Dithienyl.....	(370)
1-Ethylloxindole.....	(380)
Guaiacol.....	(65)
Hemopyrrolidine.....	(138)
Hydrazine.....	(363)
<i>m</i> -Hydroxybiphenyl.....	(394)
<i>o</i> -Hydroxybiphenyl.....	(394)
8-Hydroxyquinoline.....	(128)
Hydroxyproline.....	(137)
Hydroxythionaphthene.....	(55)
1,3-Indandione.....	(117)
<i>p</i> -Iodoacetophenone.....	(308)
α -Mercaptoacetanilide.....	(358)
<i>p</i> -Methoxyacetophenone.....	(274)
5-Methoxy-1-hydroxynaphthalene.....	(121)
2-Methylbenzimidazole.....	(395)
2-Methylbenzothiazole.....	(311)
2-Methyl-6-bromobenzothiazole.....	(311)
3-Methylcyclohexylacetophenone.....	(49)
5-Methyl-3-hydroxythionaphthene.....	(142)
7-Methyl-3-hydroxythionaphthene.....	(141)
4-Methyl-3-hydroxythionaphthene.....	(140)
Methylisopropylphenol.....	(64)
α -Methylpyrrolidine.....	(138)
α -Naphthol.....	(127)
2,1-Naphthothioindoxyl.....	(57)
α -Naphthyl ethyl ether.....	(286)
<i>p</i> -Nitrophenylhydrazine.....	(321)
Opsopyrrolidine.....	(138)
Phenyl esters of fatty acids.....	(223)

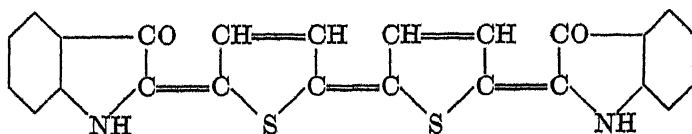
TABLE 1—Continued

SUBSTANCE REACTING WITH ISATIN OR AN ISATIN DERIVATIVE	REFERENCE
1-Phenyloxindole.....	(381)
o-Phenylphenol.....	(98)
Phyllopyrrole.....	(322)
Phyllopyrrolidine.....	(322)
Proline.....	(133)
5-Propyl-2-hydroxybiphenyl.....	(394)
Pyridine.....	(40, 139)
Pyrrole.....	(322, 324)
Quinoline.....	(412)
Quinolyl-4-acetonitrile.....	(41)
Quinoxalyl-2-acetonitrile.....	(42)
Resacetophenone.....	(60)
Resorcinol.....	(63)
Thioindoxyl.....	(161)
α -Thiol- <i>N</i> -phenylacetamide.....	(358)
Thionaphthene.....	(208)
Thymol.....	(99)
o-Toluidine.....	(58)
1-o-Tolylcarbohydrazide.....	(143)
5,6,7-Trichloro-3-hydroxythionaphthene.....	(55)
Urea.....	(62)
Xanthidrol.....	(215)

V. INDOPHENIN AND PYRROLE BLUE

In 1879 Baeyer (19) obtained a blue dye, indophenin, by treating isatin with concentrated sulfuric acid and crude benzene. V. Meyer (301) found that pure benzene did not give the indophenin reaction and that the formation of the dye was due to the presence of a new substance, thiophene, C_4H_4S . Meyer also found that other diketones containing the group $-CO-CO-$ gave the reaction. The indophenin reaction was also studied by Bauer (29) and by Liebermann and coworkers (270, 271, 273) without any definite formulation of structure or mechanism of reaction being established. Liebermann and Krauss regarded indophenin as an α -derivative of isatin (see also Ciamician and Silber (74)).

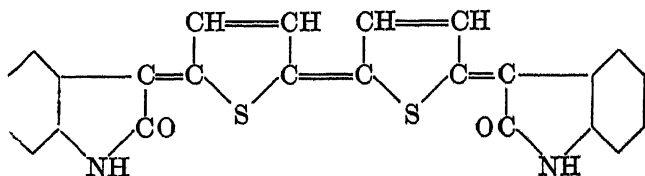
Schlenk and Blum (350), from studies of the formation of indophenin and mesoxphenin (from mesoxalic ester and thiophene) concluded that indophenin should be represented by formula I. They were able to show that the α -carbon atom of thiophene is involved in the union, since β -methyl derivatives of thiophene still give the indophenin reaction, while α -methyl derivatives do not (see also Scheiber and Schmidt (352)).



I
Indophenin

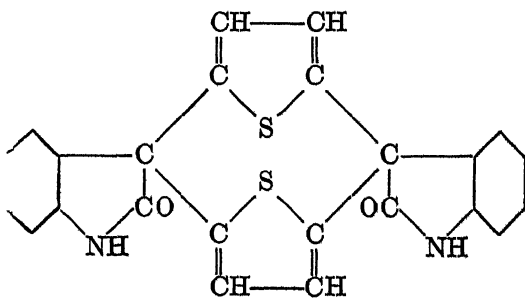
Both Heller (184) and Steinkopf (372) objected to this formulation on the grounds that isatin normally reacts through the β -carbonyl group and that the α -derivatives are obtained by indirect means only. Further, it has been shown (175) that β -derivatives of isatin do not give the indophenin reaction. On the other hand, isatin α -oxime and its ethyl ether (175) and certain other α -derivatives having the β -carbonyl group free do give the reaction (173, 335).

Both Heller (165, page 164; 184, 185) and Steinkopf (369, 370, 372) regard indophenin as a β -derivative of isatin. Heller assigned formula II to the compound, while Steinkopf first suggested formula III but later accepted the Heller formula as correct (369).



II

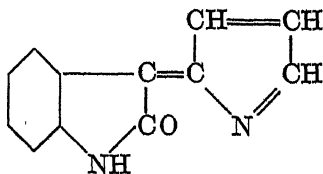
Indophenin



III

Indophenin

Similarly Steinkopf (373) regards the blue dye obtained from pyrrole and isatin by Liebermann (270, 271), Ciamician and Silber (74), and Meyer and Stadler (302) as analogous to indophenin (II) in structure. On the other hand, Pratesi (322, 324) regards pyrrole blue as having the structure IV. Molecular weight determinations by Steinkopf (373) indicate a formula having twice the molecular

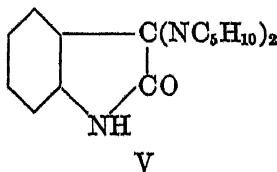


IV

Pyrrole blue

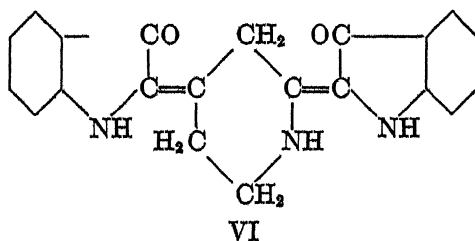
weight of IV for pyrrole blue. However, Pratesi has declined to accept the conclusions of Steinkopf, regards the double molecular weight to be due to association in solution, and holds to formula IV. In a later paper Steinkopf (374) has given further evidence in support of his formulation, and it must be stated that the weight of the evidence seems to be in his favor.

Isatin condenses with piperidine (270, 271, 356) to give two products, isatin-monopiperidide (thought to be an α -derivative by Liebermann (270)) and isatin-dipiperidide (V). The latter compound is changed on heating to isatin blue,



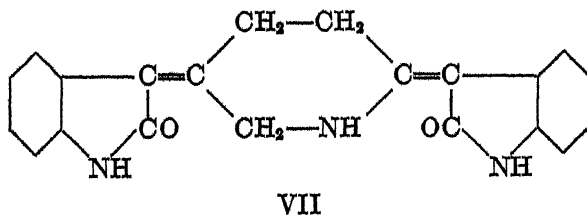
Isatindipiperidide

to which Liebermann assigned the formula VI. The formula (VII) suggested



Isatin blue

by Heller (165, page 153), which represents the dye as a β -derivative of isatin, is more acceptable than the Liebermann formula (VI).



Isatin blue

VI. CATALYTIC RÔLE IN CERTAIN ORGANIC REACTIONS

Traube (393) reported that α -aminophenylacetic acid is converted into benzaldehyde in good yield when the acid is heated with isatin in aqueous solution. Benzylamine also gave benzaldehyde in good yield on similar treatment. Abderhalden (1) has reported similarly that isatin reacts at room temperature and above with amino acids to give aldehydes with one less carbon atom. In a series of papers on organic catalysts, Langenbeck (252, 253, 254, 255, 256, 257,

258) has reported that isatin and certain isatin and oxindole derivatives possess enzyme-like activity, particularly in the dehydrogenation of amino acids.

Menke (296) has reported that isatin can be used as a microchemical reagent. The suggested utilization of isatin β -oxime as an organic precipitant (212, 213) has been mentioned.

Knecht and Hibbert (234) have reported that isatin can be determined quantitatively by titration with titanous chloride.

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POLYENE SYNTHESSES

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I. INTRODUCTION

The synthesis of polyenes is of eminent interest not only for the organic chemist but also for the physicochemist, whom it provides with hitherto inaccessible model substances required in the clarification of the laws governing the properties of homologous series, and in the study of conjugated-double-bond systems (9, 30). For the biological chemist, the same polyenes serve as models for comparison with carotenoids and other polyenic substances occurring in nature.

Discussions of the subject of the present review will be found in monographs and textbook chapters on carotenoids; polyene syntheses in particular have been treated by Bogert (6), Jones (24), and Kuhn (58). The interesting problems posed by stereoisomerism in the carotenoids and polyenes have been dealt with by Zechmeister (109).

II. METHODS

A. ALDOL AND ESTER CONDENSATIONS

In condensation reactions leading to higher polyenes at least one of the partners is generally a carbonyl compound in which one or more double bonds are

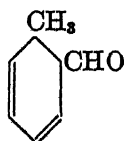
conjugated with the C=O bond. Thus, crotonaldehyde and its higher polyenic homologs ("vinyls") are the starting material for straight-chain aliphatic polyenes, whereas methylcrotonaldehyde is used as the building unit of the branched polyene chain characteristic of carotenoids. Compounds like cinnamaldehyde, citral, cyclocitral, and ionone provide cyclic and terpenoid end groups. In addition to these unsaturated carbonyl compounds, acetaldehyde and acetone themselves have been used in polyene syntheses.

1. Acetaldehyde and crotonaldehyde condensations

Acetaldehyde and crotonaldehyde undergo autocondensation in the presence of secondary amines such as piperidine. This type of Knoevenagel reaction has been used successfully for the synthesis of sorbaldehyde (I, hexadienal) (59, 68). The same mixture of acetaldehyde and crotonaldehyde also yields the higher vinyls octatrienal (II) and decatetraenal (III) (59). Aldehydes with a multiple of four carbon atoms,—namely, octatrienal (II), dodecapentaenal (IV), and hexadecaheptaenal (VI),—have been prepared by the condensation of two, three, and four molecules of crotonaldehyde with one another (5). Difficulties

- (I) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCHO}$, hexadienal
- (II) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCH}=\text{CHCHO}$, octatrienal
- (III) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCHO}$, decatetraenal
- (IV) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCHO}$, dodecapentaenal
- (V) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCHO}$, tetradecahexaenal
- (VI) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCHO}$, hexadecaheptaenal
- (VII) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCHO}$, octadecaoctaenal
- (VIII) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCH}=\text{CHCHO}$, eicosanonaenal

encountered with pure crotonaldehyde led to the discovery that secondary amines themselves are not the catalyst proper in these condensations, but that they must first form salts with the traces of crotonic acid usually present in crotonaldehyde (59). To achieve uniform results the addition of acetic acid has become customary (20, 62, 87). Cyclization occurs as an undesirable side reaction, resulting in the formation of dihydro-*o*-tolualdehyde (IX) (5, 20).

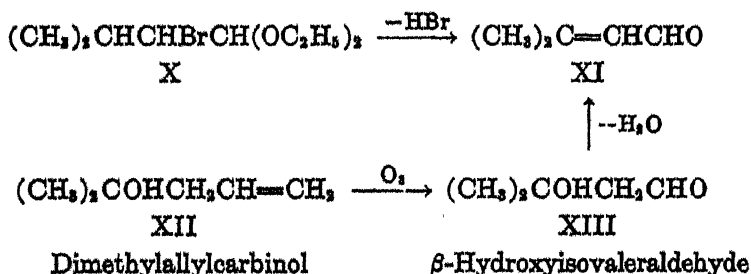


IX

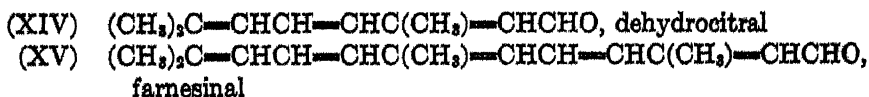
Dihydro-*o*-tolualdehyde

Mixtures of crotonaldehyde with sorbaldehyde yield tetradecahexaenal (V) and even octadecaocetaenal (VII) (91). The polymerization of five to seven molecules of crotonaldehyde was accomplished in solutions of various alcohols; the resulting products were of the general structure $C_{4n-1}H_{4n+2}(OAlk)CHO$. These alkoxy polyene aldehydes are waxy crystalline masses and are converted by complete hydrogenation into monoalkyl ethers of long-chain glycols which, in turn, may be oxidized,—e.g., to methoxylated fatty acids; the position of the methoxyl group is unknown (66). If condensation is carried out in 70 per cent ethanol at room temperature with piperidine acetate, no ethoxyl groups are introduced, but hexadecaheptaenal (VI) and some eicosanonaenal (VIII) are obtained (91). The lowest member of this series in which absorption may be observed within the range of the visible spectrum is the yellow decatetraenal (III); dodecapentaenal (IV) is orange and hexadecaheptaenal (VI) is deep red.

Polyene chains with methyl groups attached to every fourth carbon atom, such as are characteristic for carotenoids and other polyisoprenoid products of nature, may be obtained by condensation reactions with β -methylcrotonaldehyde. This intermediary may be obtained by debromination and hydrolysis of α -bromoisovaleraldehyde acetal (X) (18, 21), or by dehydration of β -hydroxyisovaleraldehyde (XIII), formed from dimethylallylcarbinol (XII) by cleavage

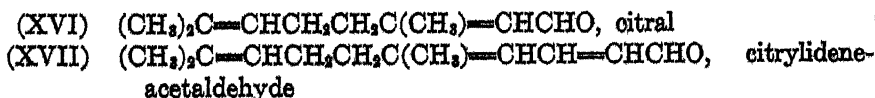


of the terminal methylene group with ozone (10). Besides cyclic byproducts, the acyclic dimer dehydrocitra (XIV) and the trimer farnesinal (XV) were obtained in analogy to the linear condensation products of crotonaldehyde by catalytic polymerization of β -methylcrotonaldehyde (5, 19, 20).



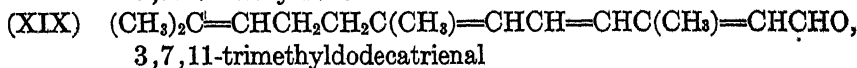
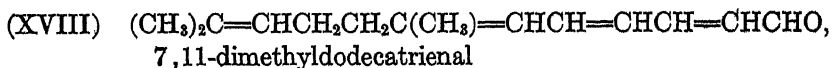
2. Condensations of terpenoid aldehydes

Citra (XVI, 3,7-dimethyloctadienal) on condensation with acetaldehyde in



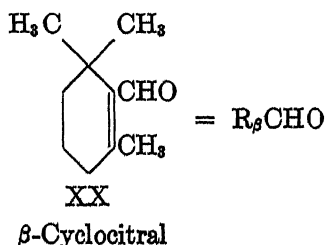
the presence of piperidine acetate (3, 59) or sodium amide (1) yields citrylideneacetaldehyde (XVII, 5,9-dimethyldecatrinal). This compound has the aroma

of pears; the reason for its non-identity with the citrylideneacetaldehyde obtained by von Braun and Rudolph (8) from citrylideneacetic acid by reduction with chromous chloride (see section 14) is not yet understood. β -Cyclocitral does not lend itself to the Knoevenagel reaction; therefore β -cyclocitrylideneacetaldehyde had to be prepared by cyclization of the semicarbazone of citrylideneacetaldehyde (39). Correspondingly, citral has been condensed with crotonaldehyde and β -methylcrotonaldehyde, yielding 7,11-dimethyldodecatrienal (XVIII) and 3,7,11-trimethyldodecatrienal (XIX) (1, 3).



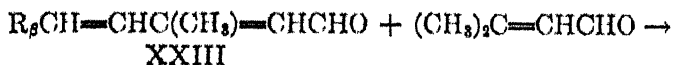
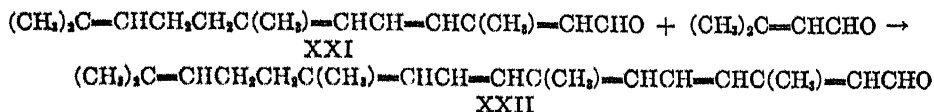
The latter compound is also designated as pseudo-ionylideneacetaldehyde. The original condensation product is not a pure substance, according to Karrer (49); it may, however, be separated through the semicarbazones into two isomeric forms designated "a" and "b". A similar condition is encountered in the case of substance XVIII; the cause of the isomerism in these cases still awaits elucidation. Because of the length of the chain, cyclization cannot be confined to the tail end of the molecule, but bicyclic condensation products are obtained, presumably in the nature of trimethyl- and tetramethyl-hexahydronaphthaldehydes (4).

The condensation of β -cyclocitral (XX) with β -methylcrotonaldehyde (25)



yielded a material which, upon reduction of the aldehyde group, gave a product resembling vitamin A in ultraviolet absorption spectrum and antimony trichloride reaction. No biological tests have been reported on this product, and it seems plausible that the polyene system present resulted from autocondensation of methylcrotonaldehyde (see Part III), an assumption (44) which reconciles these observations with the reported failure of β -cyclocitral to undergo Knoevenagel reactions. Pseudo-ionylideneacetaldehyde (XXI) and β -methylcrotonaldehyde yield under various conditions a variety of ill-defined products (XXII?) giving a blue reaction with antimony trichloride, probably for the same reason as in the preceding case (49). Likewise, the condensation of β -ionylideneacetaldehyde (XXIII), if available, with β -methylcrotonaldehyde should lead to

axerophthal (XXIV), the C_{20} -aldehyde corresponding to vitamin A (axerophthal) (72) and presumably identical with retinene (80a).

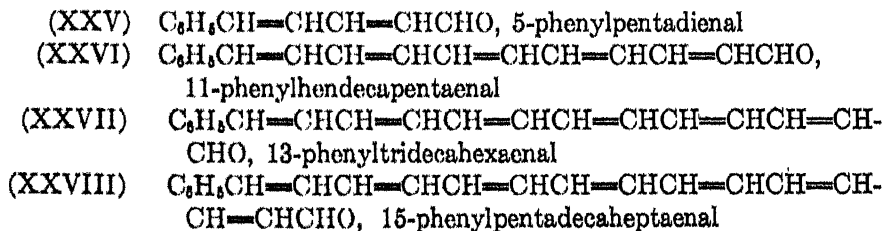


XXIV

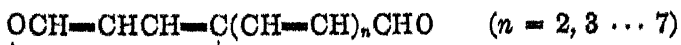
Axerophthal

3. Condensations of aromatic aldehydes

In the aromatic series, cinnamylideneacetaldehyde (5-phenylpentadienal, XXV) has been synthesized from benzaldehyde and crotonaldehyde (59, 77), 11-phenylhendecapentaenal (XXVI) from cinnamaldehyde and two molecules of crotonaldehyde, and 15-phenylpentadecaheptaenal (XXVIII) from cinnamaldehyde and three molecules of crotonaldehyde (74, 90); 13-phenyltridecahexaenal (XXVII) was prepared from XXV and two molecules of crotonaldehyde (90). An analogous series of polyene aldehydes with a terminal α -furyl group has been



prepared up to 15-(α -furyl)pentadecaheptaenal (XXIX, $n = 7$) by condensation of furfural or α -furylacrylaldehyde with acetaldehyde or crotonaldehyde in the presence of piperidine acetate (56, 90).



XXIX

4. Condensations with acetone

The condensation of unsaturated aldehydes with acetone leads to polyenic methyl ketones. In analogy to the synthesis of pseudo-ionone (XXX) from citral (XVI) with acetone (84), citrylideneacetaldehyde (XVIII) and pseudo-ionylideneacetaldehyde (XIX) ("a"-forms) condense with acetone in the presence of sodium ethoxide (4) to form 10,14-dimethyl- and 6,10,14-trimethylpentadecapentaenones (XXXI and XXXII). Acetone has also been condensed with the two isomeric γ -[2,2,6-trimethyl-1 (and 5)-cyclohexen-1-yl]- α -methyl-

- (XXX) $(\text{CH}_3)_2\text{C}=\text{CHCH}_2\text{CH}_2\text{C}(\text{CH}_3)=\text{CHCH}=\text{CHCOCH}_3$, pseudo-ionone
 (XXXI) $(\text{CH}_3)_2\text{C}=\text{CHCH}_2\text{CH}_2\text{C}(\text{CH}_3)=\text{CHCH}=\text{CHCH}=\text{CHCOCH}_3$, 10,14-dimethylpentadecapentaenone
 (XXXII) $(\text{CH}_3)_2\text{C}=\text{CHCH}_2\text{CH}_2\text{C}(\text{CH}_3)=\text{CHCH}=\text{CHC}(\text{CH}_3)=\text{CHCOCH}_3$, 6,10,14-trimethylpentadecapentaenone

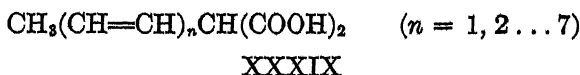
crotonaldehydes (XXXIII, XXXIV), yielding two isomeric ketones $\text{C}_{17}\text{H}_{30}\text{O}$ (XXXV, XXXVI) (36).

- (XXXIII) $\text{R}_\beta\text{CH}_2\text{CH}=\text{C}(\text{CH}_3)\text{CHO}$
 (XXXIV) $\text{R}_\alpha\text{CH}_2\text{CH}=\text{C}(\text{CH}_3)\text{CHO}$
 (XXXV) $\text{R}_\beta\text{CH}_2\text{CH}=\text{C}(\text{CH}_3)\text{CH}=\text{CHCOCH}_3$
 (XXXVI) $\text{R}_\alpha\text{CH}_2\text{CH}=\text{C}(\text{CH}_3)\text{CH}=\text{CHCOCH}_3$
 (XXXVII) $\text{R}_\beta\text{CH}=\text{CHC}(\text{CH}_3)=\text{CHCH}=\text{CHCOCH}_3$
 (XXXVIII) $\text{R}_\beta\text{CH}=\text{CHC}(\text{CH}_3)=\text{CHCH}=\text{CHC}(\text{CH}_3)=\text{CHCH}=\text{CHCOCH}_3$

Similarly, the important ketone $\text{C}_{18}\text{H}_{30}\text{O}$ (XXXVII) could be synthesized from β -ionylideneacetaldehyde, if available (41). Finally, the ketone $\text{C}_{23}\text{H}_{32}\text{O}$ (XXXVIII) was obtained in the Oppenauer oxidation of vitamin A with aluminum isopropoxide instead of the expected axerophthal (XXII), owing to the condensation of the latter with acetone (2).

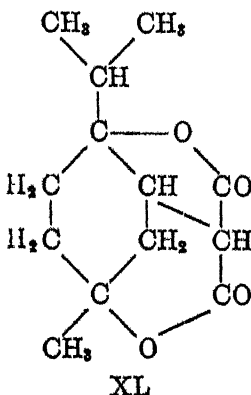
5. Malonic ester type syntheses

The condensation of acetaldehyde and diethyl malonate to yield diethyl acetylidene malonate and thence crotonic acid has been extended to the synthesis of sorbic acid (15) and of higher polyenecarboxylic acids up to hexadecaheptaenylidenemalonic acid (XXXIX, $n = 7$) (63). The higher members of this



series display increasing depth of color, the ethyl ester of the last member being deep violet. On catalytic hydrogenation of the ethyl ester of XXXIX ($n = 7$), hexadecylmalonic ester is formed, from which stearic acid is obtained by saponification and decarboxylation (67).

A peculiar reaction was observed in the attempted synthesis of citrylidene malonic acid from citral and malonic ester with pyridine (104) and saponification of the citrylidene malonic ester (28) with baryta. A substance was obtained which was insoluble in sodium carbonate solution and even in cold dilute sodium hydroxide, and tasted bitter rather than acidic. These properties were accounted for by the formation of a dilactone with two of the three double bonds eliminated. On reexamination, however, the substance proved to be fully saturated; Kuhn and Hoffer (69) were able to demonstrate the disappearance of the third double bond and to prove that "citrylidene malonic acid" is identical with (menthyl-3)malonic acid dilactone-1,4 (XL).



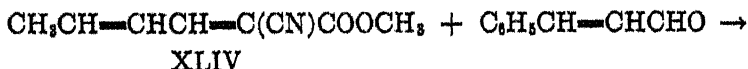
(Menthyl-3)malonic acid dilactone-1,4

The condensation of citral with ethyl cyanoacetate leads to citrylideneacyanoacetic ester; saponification of this ester with 20 per cent potassium hydroxide is accompanied by cyclization and degradation to cyclocitral (101).

Whereas diethyl malonate reacts with aldehydes only, ethyl cyanoacetate combines with ketones in the presence of acetamide in acetic acid under continuous removal of water by distillation (12). Wittig and Hartmann (105) prepared isopropylidenecyanoacetic ester (XLI) as the starting material for further condensations of isoprenoid chains. Attempts in model experiments to condense this ester with benzaldehyde or cinnamaldehyde by Cope's method (12) led to replacement of the isopropylidene group by the benzylidene or cinnamylidene group (yielding XLII or XLIII), rather than to condensation. However,

- (XLI) $(\text{CH}_3)_2\text{C}=\text{C}(\text{CN})\text{COOC}_2\text{H}_5$, isopropylidenecyanoacetic ester
 (XLII) $\text{C}_6\text{H}_5\text{CH}=\text{C}(\text{CN})\text{COOC}_2\text{H}_5$, benzylidenecyanoacetic ester
 (XLIII) $\text{C}_6\text{H}_5\text{CH}=\text{CHCH}=\text{C}(\text{CN})\text{COOC}_2\text{H}_5$, cinnamylidenecyanoacetic ester

with piperidine acetate as catalyst, condensation occurred at one of the terminal methyl groups. By similar reactions, the methyl ester of α -cyanosorbic acid (XLIV) was prepared and, in turn, condensed with cinnamaldehyde leading to the nitrile of 9-phenylnonatetraenic acid (XLV).



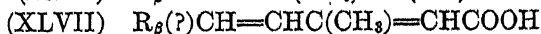
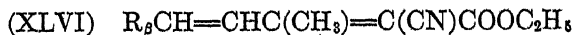
Methyl α -cyanosorbate



9-Phenylnonatetraenonitrile

The same authors (105) condensed β -ionone with ethyl cyanoacetate, using a solution of acetamide and ammonium acetate in acetic acid as catalyst. The

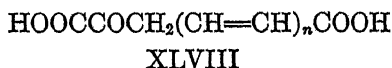
resulting ethyl β -ionylidene- α -cyanoacetate (XLVI) was saponified in three stages, yielding an ionylideneacetic acid (XLVII) identical with material prepared by Karrer (52).



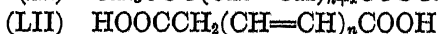
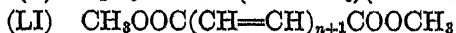
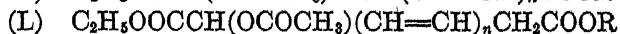
Polyenic α -keto acids may be prepared by the condensation of polyene aldehydes with pyruvic acid in strongly alkaline solution (23).

6. Oxalic ester condensations

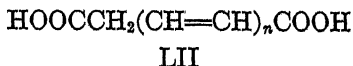
The condensation of polyenemonocarboxylic acids with diethyl oxalate leads to several series of polyenedicarboxylic acids. The reaction of diethyl oxalate and ethyl crotonate in the presence of potassium ethylate, leading to the keto acid XLVIII ($n = 1$), was discovered by Lapworth (78).



The next homolog of this series was prepared with ethyl sorbate (7). Rubidium ethylate has been recommended for these condensations, but the condensation of the higher members of the series requires the presence of pyridine (29, 62, 63, 64). The resulting oxalopolyenic esters, when treated with acetic anhydride, yield the acetyl derivatives of their enol forms (XLIX). These, in turn, upon reduction with aluminum amalgam, add one molecule of hydrogen at the ends of the conjugated-double-bond system. The resulting acetyl dihydro compounds (L), on treatment with potassium hydroxide in methanol, split off acetic acid, and the dimethyl esters of the polyenedicarboxylic acids (LI) are thus obtained.



This reaction was carried as far as tetradecaheptaene-1,14-dicarboxylic acid ($n = 6$). With the higher members, the aluminum amalgam reduction tends to replace the acetoxyl group by hydrogen, an undesirable side reaction. However, the resulting esters of polyenediacetic acids can be dehydrogenated by atmospheric oxygen in the presence of alkali (60). Oxidation of the oxalopolyenic esters with hydrogen peroxide leads to the dicarboxylic acids of the glutamic acid series which have an odd number of carbon atoms (29) (LII).



The oxalic ester method may also be utilized for the synthesis of methylated dicarboxylic acids such as β -methylmuconic acid (LIII) and 1,5-dimethylhexatrienedicarboxylic acid (LIV), from which 1,5-dimethylhexadiene-1,5-

dicarboxylic acid (LV) is obtained by partial hydrogenation with sodium amalgam (64). This acid is identical with the acid found by Hildebrandt (42) in the

(LIII) $\text{HOOCCH}=\text{CHC}(\text{CH}_3)=\text{CHCOOH}$, β -methylmuconic acid

(LIV) $\text{HOOCCH}=\text{C}(\text{CH}_3)\text{CH}=\text{CHCH}=\text{C}(\text{CH}_3)\text{COOH}$, 1,5-dimethyl-1,3,5-hexatriene-1,5-dicarboxylic acid

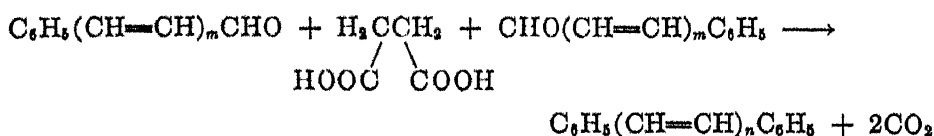
(LV) $\text{HOOCCH}=\text{C}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{CH}=\text{C}(\text{CH}_3)\text{COOH}$, 1,5-dimethyl-1,5-hexadiene-1,5-dicarboxylic acid

(LVI) $(\text{CH}_3\text{CH}=\text{C}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{CH}=\text{C}(\text{CH}_3)_2$, geraniol

urine of dogs and rabbits as a catabolic product of geraniol (LVI).

7. Perkin type condensations

A double Perkin reaction of succinic acid in the presence of lead oxide with two molecules of cinnamaldehyde (LVII) resulted in the formation of 1,8-diphenyloctatetraene (LX) (76). Higher homologs with two and four more vinyl groups



(LVII, $m = 1$; LVIII, $m = 2$; LIX, $m = 3$)

(LX, $n = 4$; LXI, $n = 6$; LXII, $n = 8$)

(LXI, LXII) were correspondingly obtained with 5-phenylpentadienal (LVIII = XXV) and 7-phenylheptatrienal (LIX). The synthesis of diphenylpolyenes with an odd number of double bonds was achieved by the substitution of dihydro-muconic acid (LXIII) for succinic acid. Even butadiene-1,4-diacetic acid (LXIV) may be used as center piece in this reaction (62).



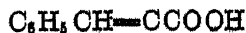
(LXIII, $n = 1$; LXIV, $n = 2$)

In order to obtain crystalline products in the condensation of benzaldehyde with crotonic acid, the latter has to be used in the form of its anhydride and triethylamine is employed as catalyst. The resulting product differs from the known stereomeric forms of cinnamylideneacetic acid (LXV) and was identified as α -vinylcinnamic acid (LXVI); the mechanism of its formation presupposes a shift of the double bond from crotonic acid to vinylacetic acid (71).



LXV

Cinnamylideneacetic acid



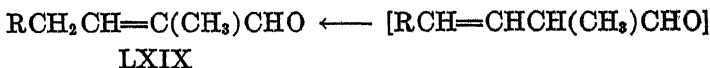
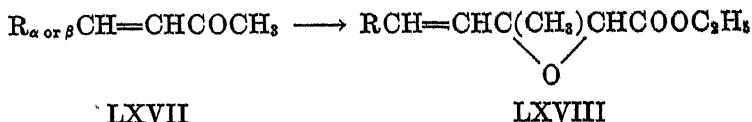
LXVI

α -Vinylcinnamic acid

8. Glycidic ester synthesis

The glycidic ester synthesis of aldehydes, when applied to the ionones (LXVII), leads to aldehydes $\text{C}_{14}\text{H}_{22}\text{O}$ (43). A careful comparison of the products

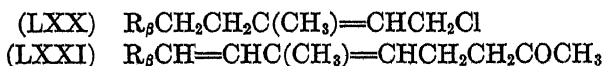
obtained both from α - and from β -ionone showed that they retained the respective position of the cyclic double bond, but that the double bond of the ionone side chain had moved in both compounds one carbon atom forward and fallen into conjugation with the aldehydic carbonyl group (LXVIII $\rightarrow$ LXIX) (34).



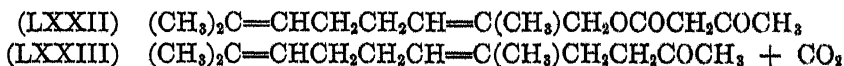
Although this reaction increases the length of the chain by one carbon only, it appears to be of significance for further syntheses.

9. Ethyl acetoacetate synthesis

This synthesis has proved of minor significance in the polyene field. It may be mentioned here, for example, that Ruzicka and Fischer (83) condensed the chloride $\text{C}_{15}\text{H}_{25}\text{Cl}$ (LXX) with ethyl acetoacetate in the course of the synthesis of tetrahydrovitamin A. Gould and Thompson (27) describe the reaction of the



corresponding triply unsaturated bromide, formulated $\text{C}_{15}\text{H}_{23}\text{Br}$, with ethyl acetoacetate and the formation of a ketone $\text{C}_{13}\text{H}_{23}\text{O}$ (LXXI). Geranylacetone has been prepared from geranyl bromide by an acetoacetic ester synthesis (88). Geranylacetone may also be obtained by rearrangement of the geranyl ester of acetoacetic acid; such esters are prepared from the sodium compound of the unsaturated alcohol plus diketene (54a) (LXXII, LXXIII).



B. SYNTHESSES WITH METALLOORGANIC COMPOUNDS

10. Reformatsky syntheses

The Reformatsky reaction of carbonyl compounds with ethyl bromoacetate and zinc has been applied to a number of polyenic methyl ketones such as crotonylideneacetone, cinnamalacetone, and 6-methyl-3,5-heptadiene-2-one (21, 70). The resulting β -hydroxy esters yield, depending on the conditions of dehydration, *cis*- or *trans*-isomers of β -substituted crotonic acids. *Cis-trans* pairs of 3-methyloctatrienic acid (LXXIV), 3-methyl-7-phenylheptatrienic acid (LXXV), and 3,7-dimethyloctatrienic acid (LXXVI) were thus obtained from the three ketones mentioned above. The higher melting form of LXXVI was identical with natural dehydrogeranic acid (11). It was found (22) that unsaturated β -hydroxy esters are simultaneously decarboxylated and dehydrated

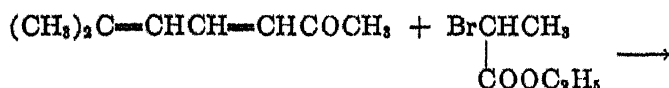
upon saponification; thus polyenes are formed besides the expected acids. Decarboxylation occurred more readily when a number of conjugated double bonds were present. The hydrocarbons phenylpentadiene, 2-methylheptatriene (from LXXIV), and 2,6-dimethylheptatriene (from LXXVI) were prepared in this manner (21).

(LXXIV) $\text{CH}_3\text{CH}=\text{CHCH}=\text{CHC}(\text{CH}_3)=\text{CHCOOH}$, 3-methyloctatrienic acid

(LXXV) $\text{C}_6\text{H}_5\text{CH}=\text{CHCH}=\text{CHC}(\text{CH}_3)=\text{CHCOOH}$, 3-methyl-7-phenylheptatrienic acid

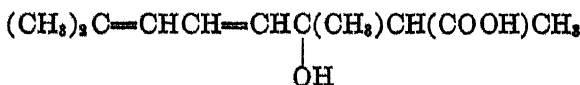
(LXXVI) $(\text{CH}_3)_2\text{C}=\text{CHCH}=\text{CHC}(\text{CH}_3)=\text{CHCOOH}$, 3,7-dimethyloctatrienic acid

A Reformatsky reaction of 2-penten-1-al with ethyl α -bromopropionate was used in the synthesis of Hildebrandt's acid (LV) (64). The condensation of 6-methyl-3,5-heptadiene-2-one (LXXVII) with α -bromopropionic ester followed by simultaneous decarboxylation and dehydration, led to alloöcimene (LXXVIII $\rightarrow$ LXXIX) (21).



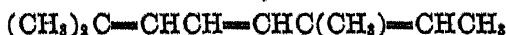
LXXVII

6-Methyl-3,5-heptadiene-2-one



OH

LXXVIII

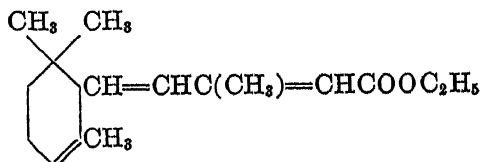


LXXIX

Alloöcimene

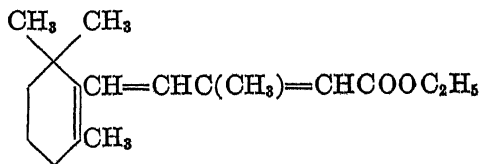
The condensation of ethyl bromoacetate with β -ionone results in a mixture of isomeric ethyl ionylideneacetates (13, 40, 52, 72). Upon saponification a crystalline acid of m.p. 125°C. was obtained in poor yield (52; cf. 105). Ethyl α -ionylideneacetate was prepared by the same method (93); the ultraviolet absorption spectrum (95) does not contradict the formulation LXXX with two ethylene bonds in conjugation with the carbethoxyl group. However, the Reformatsky condensation of pure β -ionone under the same conditions yielded a product closely resembling the α -ester in boiling point, refractive index, and ultraviolet absorption; the spectrum definitely excluded the formulation LXXXI with a total of four conjugated double bonds. The slight differences in ultraviolet absorption between the two esters and also two other isomers, prepared

by the use of magnesium instead of zinc, may be due to *cis-trans* isomerism (95; cf. 109).



LXXX

Ethyl α -ionylidene-acetate



LXXXI

Ethyl β -ionylidene-acetate

An extension of the Reformatsky reaction to ethyl γ -halogenocrotonate was described with benzaldehyde and cyclohexanone as model substances, yielding, upon dehydration of the primary reaction product, cinnamylideneacetic acid (LXXXII) and cyclohexylidenecrotonic acid (LXXXIII). Similarly, ethyl γ -bromo- β -methylcrotonate was observed to undergo a Reformatsky reaction with benzophenone (92) and with β -cyclocitral (95), but dehydration of the resulting δ -hydroxy acids (such as LXXXIV from cyclocitral) met with difficulties (cf. 25a).

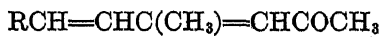
(LXXXII) $\text{C}_6\text{H}_5\text{CH}=\text{CHCH}=\text{CHCOOH}$, cinnamylideneacetic acid

(LXXXIII) $\text{C}_6\text{H}_{10}=\text{CHCH}=\text{CHCOOH}$, cyclohexylidenecrotonic acid

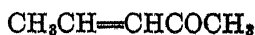
(LXXXIV) $\text{R}_2\text{CHOHCH}_2\text{C}(\text{CH}_3)=\text{CHCOOH}$

11. Zinc and cadmium alkyls

Methylzinc iodide was used to extend the side chain of " β "-ionylideneacetyl chloride to ionylideneacetone ("eu-ionone", LXXXV) (51). The application of cadmium alkyls according to the method of Gilman (26) and deBenneville (14) for this and similar purposes seems promising in view of the authors' experiments with the condensation of crotonyl chloride and dimethylcadmium resulting in the formation of 3-penten-2-one (LXXXVI).



LXXXV

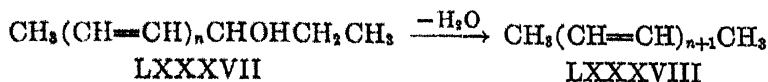


LXXXVI

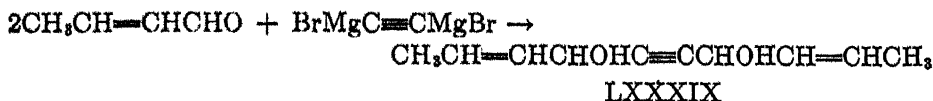
12. Grignard reactions

The polyene aldehydes react as expected with alkylmagnesium halides and yield the respective secondary alcohols (LXXXVII); these are dehydrated preferably by a 1 or 2 per cent solution of toluenesulfonic acid in ether (65). Octatriene, decatetraene, and tetradecahexaene were obtained by this procedure

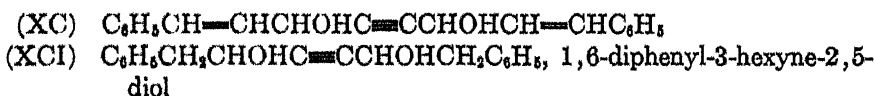
(LXXXVIII). By the use of benzylmagnesium chloride one can extend this reaction to the preparation of polyenes with terminal phenyl groups: e.g., 1,12-diphenyldodecahexaene (74). Another method for the preparation of diphenyl-



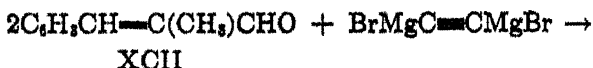
polyenes has been mentioned previously (section 7); a third alternative utilizes a di-Grignard compound as a symmetrical middle link. The dimagnesium bromides of acetylene, and especially of diacetylene, have been found superior in this respect to the corresponding ethylenic derivatives. The Grignard condensation of acetylene with unsaturated aldehydes has been used by Dupont to obtain 2,8-decadiene-5-yne-4,7-diol (LXXXIX) (16, 38; cf. 79, 96).



The application of these reactions to aromatic carbonyl compounds constitutes the third method for the synthesis of diphenylpolyenes mentioned above. Two molecules of cinnamaldehyde with dibromoacetylene yield the acetylene



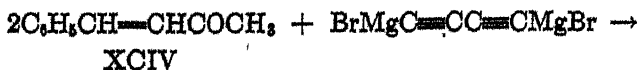
glycol (XC) (16). Two molecules of phenylacetaldehyde with dibromoacetylene give 1,6-diphenyl-3-hexyne-2,5-diol (XCI); the diphenylhexatriene obtainable from this glycol by hydrogenation and dehydration may also be obtained from the reaction of two molecules of benzaldehyde with dibromodiacetylene (74). By analogous reactions, diphenylpolyenes with branched side chains were obtained: e.g., 1,8-diphenyl-2,7-dimethyloctatetraene (XCIII) from α -methylcinnamaldehyde (XCII) and acetylene, and 1,10-diphenyl-3,8-dimethyldecapentaene (XCV) from benzalacetone (XCIV) and diacetylene by partial hydrogenation of the intermediate glycols and subsequent dehydration.



α -Methylcinnamaldehyde



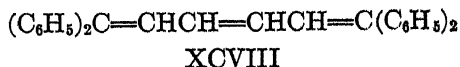
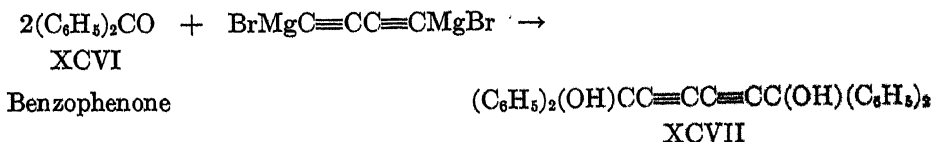
1,8-Diphenyl-2,7-dimethyloctatetraene



Benzalacetone

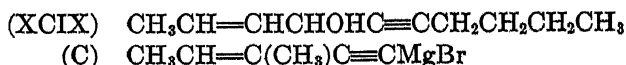


Two molecules of benzophenone (XCVI) or fluorenone lead, through a Grignard reaction with diacetylene, to the respective glycols (XCVII) and eventually to tetraphenylhexatriene (XCVIII) and di(biphenylene)hexatriene (75).



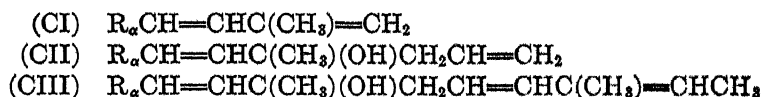
Tetraphenylhexatriene

The practical significance of one-sided Grignard reactions in the acetylene group is confined to substituted acetylenes (85, 86). Thus, the Grignard compound of 1-hexyne (butylacetylene) has been condensed with crotonaldehyde (XCIX) (37). The Grignard compound of (1,2-dimethylvinyl)acetylene (C) was condensed with butyraldehyde, crotonaldehyde, and citral (33). In the



case of all the α,β -unsaturated aldehydes mentioned, the configuration $-\text{CR}_1=\text{CR}_2\text{CHOHC}\equiv\text{C}-$ forms the center of the condensation product. Treatment with acids favors an anionotropic rearrangement to $-\text{CR}_1(\text{OH})\text{CR}_2=\text{CHC}\equiv\text{C}-$ (33, 37, 46, 79).

Various Grignard reactions have been performed with α -ionone, leading to the expected carbinols. The alcohol C₁₄H₂₃OH was obtained with methylmagnesium bromide and converted into the corresponding hydrocarbon β -cyclocitrylideneisobutene C₁₄H₂₂ (CI) (55). Similarly, allylmagnesium bromide (52) and 4-methyl-2,4-hexadienylmagnesium bromide (80) have been shown to give tertiary carbinols (CII, CIII) with α -ionone. These reactions cannot be dupli-

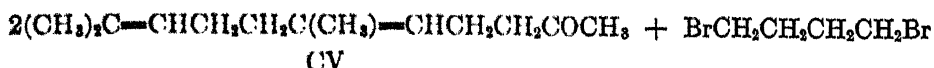


cated in the case of β -ionone, presumably owing to the presence of a second conjugated ethylene bond. Kipping and Wild (55) successfully condensed β -ionone with methyl iodide and also with bromobenzene by substituting lithium for magnesium. The unaltered presence of the β -configuration in the reaction products was confirmed by the formation of geronic acid through ozonolysis and by spectroscopic evidence.

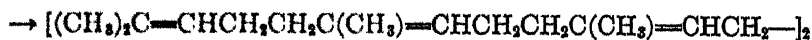
Phenyllithium was also applied to the introduction of four phenyl groups into the dimethyl ester of dihydromuconic acid, leading after dehydration of the glycol (CIV) to tetraphenylhexatriene (107) (XCVIII), identical with that obtained from benzophenone and diacetylene (74).



The attractive idea of combining two citral groups by a polyene chain, albeit a shorter one than in carotenoids, has been realized in the combination of two molecules of geranylacetone (CV) with the Grignard compound from 1,4-dibromobutane; dehydration of the resulting glycol with iodine yielded squalene (CVI). Using pseudo-ionone (CVII) instead of geranylacetone, one obtains dehydrosqualene (CVIII) (88).



Geranylacetone



CVI

Squalene



CVII

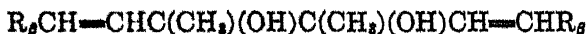
Pseudoionone



CVIII

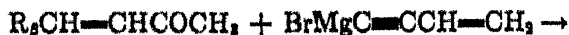
Dehydrosqualene

Again, in the case of β -ionone abnormal reactions occur; in attempts to condense β -ionone with ethylene dibromide or 1,4-dibromobutane in the presence of magnesium, the pinacol of β -ionone, $\text{C}_{20}\text{H}_{32}\text{O}_2$ (CIX), was formed by reduction



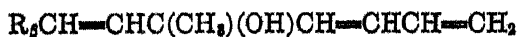
CIX

(96). The reaction of β -ionone with the magnesium compounds of vinyl ethynyl bromide (CX) and of butadienylethynyl bromide (CXIII) leads to carbinols which can be partly hydrogenated, yielding products to which the formulas CXI and CXIV were assigned.



β -Ionone

CX



CXI



CXIII

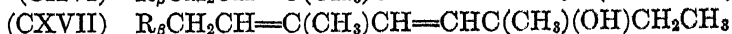
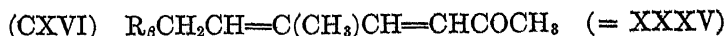


CXIV

Attempts to rearrange them into primary alcohols (CXII, CXV) failed (98).

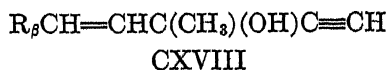


The ketone $C_{17}H_{26}O$ (CXVI), which differs in the position of the linear conjugated system from the natural carotenoids, has been converted by ethylmagnesium bromide to the carbinol CXVII (34).



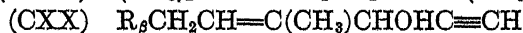
13. Acetylene condensations

Another group of condensations with acetylene is based on Nef's reaction of ketones with acetylene in the presence of sodium. The application of this procedure to β -ionone yielded amongst other products the carbinol CXVIII, which was converted by partial hydrogenation to the corresponding allyl alcohol.



Whereas no Rupe rearrangement of the ethynylcarbinol to the α,β -unsaturated aldehyde could be achieved (13, 40), the allyl rearrangement of the vinylcarbinol was described (27).

Better yields are obtained in the condensation of one molecule of aldehyde with unsubstituted acetylene by this method or with sodium amide or tertiary potassium butoxide than with the Grignard method discussed above. Sodium acetylide in liquid ammonia yields satisfactory amounts of the ethynylcarbinols derived from acrolein, crotonaldehyde, 2-methylcrotonaldehyde, tiglic aldehyde, and 2-ethyl-2-hexenal (45). Ethynylgeraniol (CXIX) was prepared by this method from citral (34). Finally, an ethynylcarbinol was obtained from the aldehyde



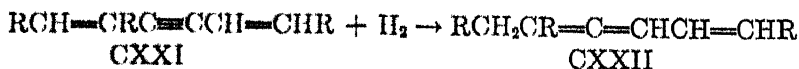
$C_{14}H_{22}O$ (LXIX). The constitution CXX is doubtful and some rearrangement seems to take place (34). These ethynylcarbinols contain the same grouping as the substances obtained by Grignard synthesis and are subject to the same anionotropic rearrangement.

C. REDUCTIONS AND OXIDATIONS

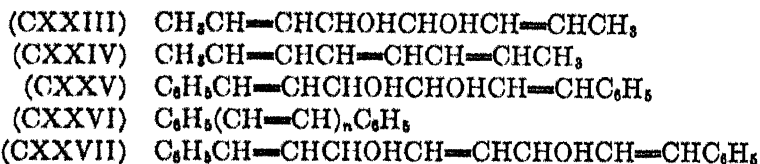
14. Reduction and hydrogenation

Many syntheses in the polyene field involve procedures of hydrogenation, both in the chain and in the functional groups. Acetylene bonds frequently have to be reduced to ethylene bonds. This end may be achieved by a variety of methods with differing degrees of specificity. Metallic sodium in alcohol, Raney nickel, palladium on various carriers, and platinum have all been used for this purpose. The uptake of hydrogen must be controlled in order to interrupt hydrogenation at the ethylene level. An unusually specific catalyst for the partial hydrogenation of triple bonds is an iron catalyst, prepared from an iron-aluminum alloy in analogy to the method used for Raney nickel (81, 100). Vinylacetylenes are thus easily converted into the corresponding dienes. In the

case of symmetrical divinylacetylenes, the resulting hexatrienes seem to undergo a rearrangement whereby one of the original double bonds is moved one step closer to the partly hydrogenated acetylene bond, resulting in a vinyl-allene configuration (99) (CXXI, CXXII).



The removal of the elements of two molecules of water from a polyenic glycol leads to a polyene with two more double bonds. Frequently, however, 1,2-, 1,4-, or 1,6-glycols are available as intermediates. These require the removal of two hydroxyls only and the introduction of a single additional ethylene bond. Obviously, such a reaction is in the nature of a reduction (*cf.* CXXIII and CXXIV) (102). A reagent specific for this reduction has been found in phosphorus diiodide, P_2I_4 (76), by means of which dihydrocinnamoin (CXXV) has



been converted into diphenylhexatriene (CXXVI, $n = 3$) and 1,8-diphenyl-1,4,7-octatriene-3,6-diol (CXXVII) into diphenyloctatetraene (CXXVI, $n = 4$). The former instance illustrates the synthesis of a symmetrical polyene with an odd number of double bonds.

Phosphorus diiodide in pyridine solution produces an unusual effect when reacting on a substance such as 1,1,6,6-tetraphenyl-2,4-hexadiyn-1,6-diol (CXXVIII), in which no hydrogen atoms are available for the removal of two molecules of water. Tetraphenylhexapentaene is formed by this reducing agent, and also by chromous chloride or by hydriodic acid; this substance (CXXIX) is a representative of the cumulenes, interesting substances with a chain of contiguous double bonds (75).



The synthesis of longer polyene chains from two equal fragments without a middle piece can be afforded by certain reductive procedures. Dihydrocinnamoin (CXXV) is obtainable from cinnamaldehyde by reduction with zinc. In analogy to the reductive condensation of thiobenzaldehyde through the free benzylidene radical to stilbene, thio- and seleno-polyene aldehydes may be dimerized by various reducing agents; thus, 1,22-diphenyldocosahendecaenal (CXXXI), a hydrocarbon of purple-black color with metallic lustre and having a melting point of 318°C ., was prepared from 11-phenylhendecapentaenal



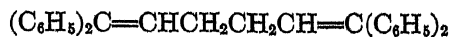
11-Phenylhendecapentaenal 1,22-Diphenyldocosahendecaene

(CXXX) (58). An unusual condensation of two molecules of allyl chloride to hexatriene and of two molecules of methallyl chloride to 2,5-dimethylhexatriene by means of sodium amide in liquid ammonia with comparatively good yields has been described (54).

The particular suitability of aldehydes for polyene syntheses frequently requires the reduction of carboxyl groups to aldehyde groups in the presence of double bonds. The reduction method of von Braun and Rudolph (8) has been tried for the conversion of ethyl ionylideneacetate to ionylideneacetaldehyde with indifferent results (72). The ester is treated with the Grignard compound of *o*-toluidine, and the resulting *o*-toluidide is converted by phosphorus pentachloride into the imide chloride, which in turn is reduced by chromous chloride to the aldehyde (see, however, 53, 34). Another attempt to prepare ionylideneacetaldehyde,—namely, by dry distillation of the mixed barium salts of ionylideneacetic acid and formic acid,—led to a carbonyl compound, which was not identical with the expected ionylideneacetaldehyde (9), but which was recently shown to be α -ionone (93). Analogous scissions of ethenoid linkages in the α,β -position to carbonyl groups have been described: e.g., in the breakdown of citral into methylheptenone and acetaldehyde (103).

15. Oxidation and dehydrogenation

In those instances where an aldehyde has to be derived from the corresponding primary alcohol, the use of ketones and aluminum alcoholate is indicated. Since acetone is prone to condense with the unchanged aldehyde, the use of diisopropyl ketone has been recommended (31, 32). In the case of natural vitamin A, this reaction leads to the formation of axerophthal (XXII) or, when using acetone, to the formation of the ketone $C_{28}H_{32}O$ (XXXVIII) (2). Conversely, aluminum isopropoxide in isopropyl alcohol is the reagent of choice for the reduction of polyenic aldehydes to the respective alcohols (1, 19, 20, 72, 82). Polyencarboxylic acids have been prepared from the corresponding aldehydes by oxidation with silver oxide (5, 82). The preparation of acids of the glutaconic series (LII) by the oxidation of oxalopolyenic esters (XLVIII) with hydrogen peroxide has been mentioned in section 6 (29). In the same section, reference has been made to the dehydrogenation of polyenediacetic acids to polyenedicarboxylic acids with one more double bond by atmospheric oxygen in the presence of alkali (60). Finally, 1,1,6,6-tetraphenyl-1,5-hexadiene (CXXXII) has been dehydrogenated to the corresponding triene (XCVIII) by selenium dioxide (87).



CXXXII

1,1,6,6-Tetraphenyl-1,5-hexadiene

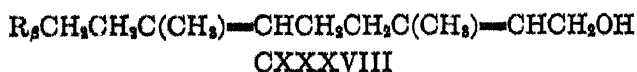
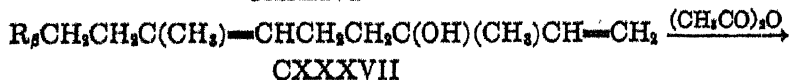
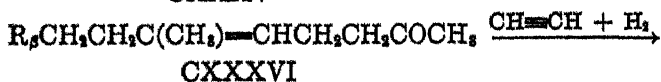
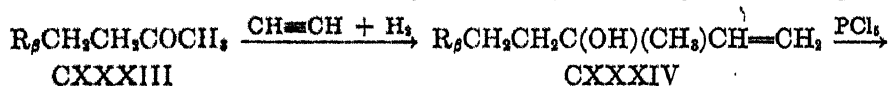
III. ATTEMPTS AT SYNTHESIS OF VITAMIN A

One of the most interesting practical applications of polyene syntheses would be a successful synthesis of vitamin A. The two main obstacles in this task, as well as in the synthesis of other natural carotenoids, are the conjugated-double-

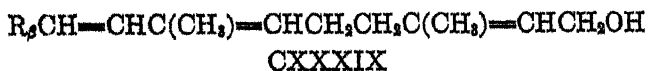
bond system and the lateral methyl groups, which necessitate the use of ketones instead of the more reactive aldehydes. The former difficulty,—namely, that offered by the conjugated-double-bond system,—is circumvented in syntheses of perhydrogenated or at least partly hydrogenated products, which have played an important part in the confirmation of the structural formulas proposed for vitamin A and other carotenoids.

The perhydro derivatives of the acyclic carotenoid dicarboxylic acids crocetin and norbixin (bixin is the monomethyl ester of norbixin),—namely, perhydrocrocetin and perhydronorbixin,—have been synthesized by sequences of classical reactions (6, 47, 48). For the synthesis of squalene see section 12 (CVI, CVIII). The mono-unsaturated natural alcohol phytol, the alcoholic component of chlorophyll, has been synthesized by F. G. Fischer and Loewenberg (17). A Wurtz synthesis of dihydrophytyl bromide yielded perhydrolycopene (50). Des-crocetin, tetradecaheptaene-1,14-dicarboxylic acid, has been prepared by Kuhn and Grundmann (63).

Perhydrovitamin A was synthesized by Karrer, Morf, and Schoepp (51). In this case, as in all cases mentioned above, the identity of the synthetic perhydro derivative with the hydrogenation product of the corresponding natural substance was established. Ruzicka and Fischer (83), starting from dihydro- β -ionone, synthesized tetrahydrovitamin A according to formulas CXXXIII to CXXXVIII. Neither the tetrahydrovitamin, which may also be designated



as 1- β -cyclogeranylgeraniol, nor the dihydrovitamin A (CXXXIX) of Gould and Thompson (27), showed any vitamin A activity. The latter authors used

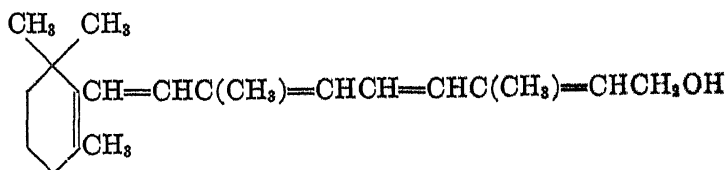


Tetrahydrovitamin A

essentially the same sequence of reactions as Ruzicka, but started from ionone instead of dihydroionone.

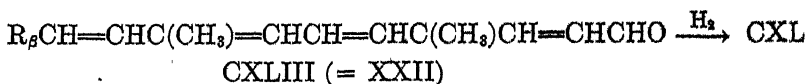
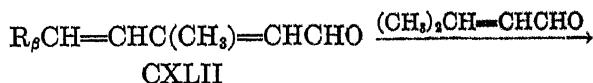
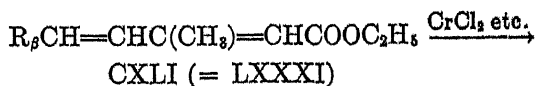
The numerous attempts to prepare a biologically active alcohol with twenty carbon atoms and five conjugated double bonds according to the specifications

of formula CXL have not yet met with success. Kuhn and Morris (72) claim the synthesis of a mixture containing 7 per cent of biologically active material by the following steps:



CXL

Vitamin A



Except for their own scantily reported biological tests, all evidence gathered in the attempted repetition of the work of Kuhn and Morris speaks against their claims. First, the β -configuration of the ethyl ionylideneacetate (CXLII), obtained by the Reformatsky condensation of β -ionone with ethyl bromoacetate, is highly questionable (93, 95; cf. 108). Second, the reduction of this ester by the chromous chloride method, as described by Kuhn and Morris could not be reproduced by Karrer (53), Heilbron (34, 35), Krauze (57), or the authors. Finally, the formation of a chromogen for the Carr-Price reaction in a condensation involving the use of β -methylcrotonaldehyde is ambiguous, since here, as in the case of the attempted synthesis of Fuson and Christ (25), autopolymerization of methylcrotonaldehyde offers a sufficient explanation for a blue color reaction with antimony trichloride (see section 2).

Obviously, the preparation of authentic β -ionylideneacetaldehyde (CXLII) would present one of the most important stepping stones in the synthesis of vitamin A. Neither the above synthesis by Kuhn and Morris, nor the method of Davies, Heilbron, and coworkers with barium formate (13), nor the insufficiently supported Grignard reaction of β -ionone with diethyl bromoacetal (57) have led to success in this direction. The acetylene method of Ruzicka and Fischer (83), which works well in the dihydroionone series, gave very poor yields in the case of β -ionone itself (27). The alcohol $R_\beta\text{CH}=\text{CHC}(\text{CH}_3)=\text{CHCH}_2\text{OH}$ (CXLIV) formed as an intermediate in the latter synthesis, is a potential source of ionylideneacetaldehyde by Oppenauer dehydrogenation.

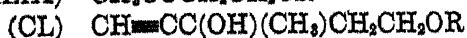
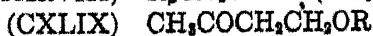
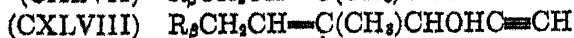
In view of the objections against the condensation of β -ionylideneacetaldehyde with methyletonaldehyde, an alternative synthesis has been considered (40): Condensation of ionylideneacetaldehyde with acetone would lead to the isoprenolog of ionone, the ketone $C_{18}H_{26}O$ (XXXVII) (section 4). An authentic synthesis of this ketone depends on the preparation of β -ionylideneacetaldehyde. It would form an appropriate starting material for another acetylene synthesis and allylic rearrangement leading to vitamin A, analogous to the reactions $CXXXVI \rightarrow CXXXVIII$. The extended Reformatsky condensation of ethyl γ -iodocrotonate with β -ionone has been suggested by Jones as an alternative (44). This would lead to the acid $C_{17}H_{24}O_2$ (CXLV), the chloride of which may be combined with methylcadmium to yield the ketone $C_{18}H_{26}O$ (XXXVII) mentioned above.



CXLV

The unexpected tendency of the conjugated-double-bond system of β -ionone and its derivatives to undergo rearrangement into α -ionone and its respective derivatives under a variety of experimental conditions complicates most of the proposed synthetic schemes and necessitates the checking of the steric configuration by time-consuming physical and chemical tests at every step. The resolution of α -ionone into its enantiomers (94) offers the possibility to use optically active α -ionone as an indicator for the occurrence or otherwise of such rearrangements. The only authenticated β -ionone derivative with more than thirteen carbon atoms is β -cyclocitrylideneisobutene (*cf.* CI), obtained by the reaction of methyl lithium upon β -ionone. Hence Kipping and Wild, in a preliminary communication (55), allude to a synthesis of vitamin A by condensation of β -ionone with the methyl ether of 6-bromo-3-methyl-2,4-hexadien-1-ol, $BrCH_2CH=CHC(CH_3)=CHCH_2OCH_3$ (CXLV), yielding the methyl ether of vitamin A.

Another possible scheme for the synthesis of vitamin A has been envisaged by Heilbron, Johnson, Jones, and Spinks (34) as illustrated by the following formulas: The ethynylcarbinol CXLVIII (= CXX, section 13) may be obtainable from the aldehyde CXLVII (= LXIX, section 8), and could then be condensed with a derivative of butanolone (CXLIX), with a protected hydroxyl group. As an alternative, the aldehyde CXLVII may be condensed with a correspondingly protected derivative of the ethyl carbinol CL.



In either alternative one may anticipate difficulties in the dehydration and in the anionotropic rearrangement of the primary condensation products.

IV. SYNOPSIS OF SYNTHETIC POLYENES

Classification	Method	References
Aliphatic hydrocarbons ...	(a) Dehydration of secondary or tertiary carbinols obtained by Grignard reaction from carbonyl compounds (b) Thermal dehydration of glycols (c) Simultaneous dehydration and decarboxylation of hydroxy acids (d) Wurtz reaction	(21, 65) (88, 102) (21) (54)
Terpenoid polyenes	(a) Dehydration as above (a) (b) Simultaneous dehydration and decarboxylation as above (c)	(22, 55) (21, 22)
Aromatic hydrocarbons with even number of double bonds.....	(a) Dehydration of carbinols (b) Geminal Perkin reaction of aromatic aldehydes with succinic acid or its vinylogs (c) Grignard reaction of unsaturated aromatic aldehydes with benzyl halogenide (d) Geminal Grignard reaction of aromatic aldehydes with an aliphatic middle piece (e) "Dehydroxylation" of 1,2- or 1,8-glycols with phosphorus diiodide	(65) (5, 76) (74) (16, 74) (76)
Aromatic hydrocarbons with odd number of double bonds.....	(a) Geminal Perkin reaction of aromatic aldehydes with dihydromuconic acid (b) "Dehydroxylation" of 1,4-glycols (c) Dehydrogenation of less unsaturated hydrocarbons with selenium dioxide (d) Reductive condensation of polyenic aldehydes (e) Aryl lithium condensation of dihydro muconic ester and dehydration of resulting ditertiary glycol	(62) (76) (87) (58) (107)
Cumulenes.....	Dehydration of acetylenic glycols by various reagents	(75)
Primary alcohols.....	(a) Ponndorf hydrogenation of aldehydes (b) Allylic rearrangement	(1, 19, 20, 72, 82) (27, 33, 37, 46, 79, 92)

Classification	Method	References
Secondary and tertiary alcohols	(a) Grignard condensation of aldehydes and ketones (b) Acetylene condensation of aldehydes and ketones	(37, 52, 55, 65, 80) (34, 45)
Glycols	(a) Reductive condensation of two molecules of aldehyde (b) Geminal Grignard condensation with dibromoacetylene and partial hydrogenation (c) Geminal double Grignard condensation with dicarboxylic esters	(58) (16, 38, 79, 96) (74)
Aldehydes	(a) Oppenauer dehydrogenation (b) von Braun reduction of ester <i>via</i> imide chloride (c) Thermal reaction of barium salt with barium formate (d) Aldol condensations	(3) (8, 34, 53, 72) (9, 34, 93) (1, 3, 5, 19, 20, 39, 50, 50, 62, 68, 77, 87, 90, 91)
Ketones	(a) Condensation with acetone (b) Condensation with ethyl acetoacetate (c) Condensation of acyl chlorides with methylzinc or methylleadmium	(2, 4, 36, 84) (27, 83, 88) (14, 26, 51)
Monocarboxylic acids. .	(a) Oxidation of aldehydes (b) Malonic ester synthesis with aldehydes and decarboxylation (c) Cyanoacetic ester condensation, followed by saponification and decarboxylation (d) Reformatsky synthesis (e) Elimination of CO from α -keto acids	(5, 82) (15, 63) (12, 105) (13, 21, 40, 52, 64, 70, 72, 93, 95, 105) (23)
Dicarboxylic acids; substituted malonic acids .	Malonic ester synthesis	(15, 63)
Dicarboxylic acids; fumaric acid series	Oxaloacetic ester synthesis	(7, 29, 60, 62, 63, 64, 78)
Dicarboxylic acids; glutamic acid series	Oxidation of oxalo acids with hydrogen peroxide	(20)

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COORDINATION COMPOUNDS OF BORON TRICHLORIDE. I

A REVIEW

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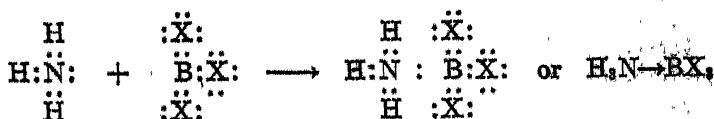
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I. INTRODUCTION

In an earlier paper (3) the coordination compounds of boron trifluoride were reviewed. Many molecular compounds of boron trifluoride have been reported, and it is of interest to compare the number of these with the number of similar compounds formed with boron trichloride. Hackspill (29) and Moissan (45), in their reviews of the chemistry of boron trichloride, report only a few coordination compounds of boron trichloride. A survey of the literature reveals that boron trichloride forms fewer coordination compounds than boron trifluoride. This may be accounted for in several ways. First, boron trichloride has a larger molecule than boron trifluoride. The chlorine atom is larger than the fluorine atom; therefore the chlorine nuclei are farther from the boron atom in boron trichloride and thus less firmly attached than are the fluorine atoms in boron trifluoride. Evidence of this is abundant in the reactions of these two boron halides with organic compounds, in which boron trichloride readily splits out hydrogen chloride to form organo-boron chloride compounds, while boron trifluoride readily coordinates with organic compounds to form molecular compounds. Also, owing to the difference in size of the chlorine and fluorine atoms, steric hindrance to the formation of coordination compounds might be more readily encountered with boron trichloride than with boron trifluoride.

Secondly, in molecular compounds the boron halide molecule is electronegative, owing to the acceptance of a pair of electrons by the boron atom. For example:



Since fluorine is more electronegative than chlorine, boron trifluoride will be more electronegative than boron trichloride; thus boron trifluoride should form molecular compounds with greater ease.

There are only seven elements which in their compounds have been reported to be donors to the boron atom of boron trichloride. These elements are nitrogen, phosphorus, and arsenic in Group V of the Periodic Table, oxygen and sulfur in Group VI, and fluorine and chlorine in Group VII. All of these elements except arsenic are found to be donors to boron trifluoride (3). In addition, carbon and argon are donors to boron trifluoride.

The greatest difference between boron trichloride and boron trifluoride is found in their reactions with organic compounds, as described above.

In this paper the coördination compounds of boron trichloride will be discussed with respect to the group in the Periodic Table of which the donor atom is a member.

II. GROUPS 0 TO IV

No research has been reported with the rare gases in Group 0 analogous to the argon-boron trifluoride study of Booth and Willson (5). It would be interesting to see if argon or krypton will coördinate with boron trichloride.

The literature reveals no coördination compound studies involving the members of Groups I to IV and boron trichloride. The same situation is found with boron trifluoride, although in this case it is postulated that certain organic compounds and boron trifluoride form intermediate coördination compounds in which the carbon atom is the donor (3).

III. GROUP V

Nitrogen, phosphorus, and arsenic in their respective compounds serve as donors to the boron atom of boron trichloride in forming coördination compounds. The nitrogen atom in ammonia, substituted ammonias, and cyanides coördinates with boron trichloride. Phosphine and the chlorides of phosphorus are the compounds in which the phosphorus atom serves as the donor to the boron atom of boron trichloride. Arsine is reported to form a molecular compound with boron trichloride. In this compound the arsenic is the donor atom.

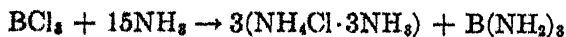
A. Nitrogen

According to Berzelius (1) 1 volume of boron trichloride combines with 1.5 volumes of ammonia to form the white solid $3\text{NH}_3 \cdot 2\text{BCl}_3$. Martius (41) prepared this compound by passing dry ammonia into cooled liquid boron trichloride. The compound does not fume in air, is less volatile than ammonium chloride, and sublimes without decomposition. It reacts with water to produce hydrochloric acid, ammonium chloride, and ammonium borate.

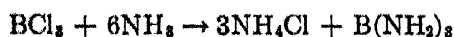
Besson (2) reported that ammonia displaces phosphine from the compound $\text{PH}_3 \cdot \text{BCl}_3$ at 8°C . to produce $9\text{NH}_3 \cdot 2\text{BCl}_3$. This compound changes slightly when exposed to air, but does not lose ammonia below 50°C . It reacts rapidly with water, producing an alkaline solution. Joannis (33) thought this substance

to be a mixture of $6\text{NH}_4\text{Cl} + \text{B}_2(\text{NH})_3$ and proposed that the above-mentioned results were due to the complexity of the reactions taking place. He concluded that mixtures of boron-substituted ammonias with ammonium chloride had been formed instead of coördination compounds.

By bubbling hydrogen through cooled ($0^\circ\text{C}.$) liquid boron trichloride and then into cooled liquid ammonia Joannis (33) studied the reaction between ammonia and boron trichloride between $-70^\circ\text{C}.$ and $-50^\circ\text{C}.$ A white precipitate formed immediately in the liquid ammonia. The temperature of the bath was then raised to $-23^\circ\text{C}.$ and the excess ammonia evaporated. Examination of the product showed that fifteen molecules of ammonia had been fixed. At $-23^\circ\text{C}.$ the following reaction was thought to occur:

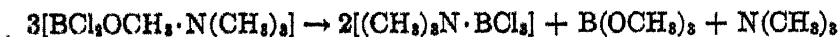


Nine molecules of ammonia were evolved when the product was heated to $0^\circ\text{C}.$ The vapor pressure of the resulting products was the same as that of the compound $\text{NH}_4\text{Cl} \cdot 3\text{NH}_3$. During this reaction neither hydrogen nor nitrogen was evolved; therefore six molecules of ammonia rearranged to form three amido groups and three ammonium groups:



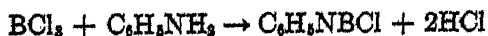
All of this early work on the reaction between ammonia and boron trichloride seems questionable. If coördination compounds are formed, why has $\text{NH}_3 \cdot \text{BCl}_3$ not been found? Boron trifluoride forms $\text{NH}_3 \cdot \text{BF}_3$, $2\text{NH}_3 \cdot \text{BF}_3$, and $3\text{NH}_3 \cdot \text{BF}_3$ (3).

Substituted ammonias, such as trialkylamines, have been reported to coördinate with boron trichloride. Wiberg and Sütterlin found that boron trichloride and trimethylamine react at $-40^\circ\text{C}.$ to yield $(\text{CH}_3)_3\text{N} \cdot \text{BCl}_3$. This compound melts at $243^\circ\text{C}.$ and is stable up to $250^\circ\text{C}.$ (69). These authors also prepared this compound by the thermal decomposition of $\text{BCl}_3\text{OCH}_3 \cdot \text{N}(\text{CH}_3)_3$:



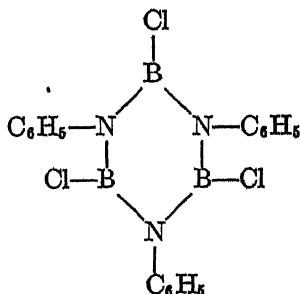
The corresponding ethyl derivative, $(\text{C}_2\text{H}_5)_3\text{N} \cdot \text{BCl}_3$, has been mentioned by Quill (49), but no experimental details were given. Gustavson (23, 27) investigated the reaction of ethylamine and boron trichloride. The product obtained melts and sublimes when heated. At $200^\circ\text{C}.$ it decomposes, yielding ethyl chloride and boron nitride.

In the aromatically substituted ammonias, Rideal (52) has reported that one mole of aniline reacts with one mole of boron trichloride, producing a white solid, melting at $175^\circ\text{C}.$ and having the composition $\text{C}_6\text{H}_5\text{NCl}$. The reaction can be expressed as follows:



Jones and Kinney (34) prepared the compound $\text{C}_6\text{H}_5\text{NH}_2 \cdot \text{BCl}_3$ by the reaction of aniline and boron trichloride in benzene at $-15^\circ\text{C}.$ The coördination compound decomposes in air. It begins to melt at $100^\circ\text{C}.$ but decomposes at $120^\circ\text{C}.$

When the compound is heated in boiling benzene, boron trichloride is evolved. Triphenyltrichlorotriboron nitride forms in the benzene filtrate, crystallizing in long needles which sinter at 255–260°C. and melt with decomposition at 265–270°C. The determination of the molecular weight indicates that the substance is trimolecular. The authors propose the structure:



It is possible to write the formula of an isomer which would be a coordination compound of boron trichloride, $(\text{C}_6\text{H}_5\text{N})_3\text{B}_2 \cdot \text{BCl}_3$. However, the cyclic formula is favored by the mode of preparation of the compound. With water, the three chlorine atoms of the compound are replaced by three hydroxyl groups. Complete hydrolysis yields aniline and boric acid.

Jones and Kinney (34) studied the reaction of dimethylaniline with boron trichloride in benzene at -15°C . A colorless solid, $\text{C}_6\text{H}_5\text{N}(\text{CH}_3)_2 \cdot \text{BCl}_3$, is obtained which softens at 125–130°C. and melts completely at 146°C. The melt crystallizes at 144–145°C. The solid fumes in air and is insoluble in cold water.

p-Toluidine-boron trichloride, $p\text{-CH}_3\text{C}_6\text{H}_4\text{NH}_2 \cdot \text{BCl}_3$, has been reported by Kinney and Kolbezen (35), who prepared it in benzene as a solvent. It melts with evolution of hydrogen chloride at 159–160°C. By distilling all of the hydrogen chloride out of the benzene filtrate, tri-*p*-tolyltrichlorotriboron nitride ($p\text{-CH}_3\text{C}_6\text{H}_4\text{NBCl}$)₃ is isolated. Kinney and Kolbezen reported that this compound softens at 304°C. and melts at 308–309°C. with decomposition. Its structure can be formulated as a cyclic compound or as an addition compound, in analogy to triphenyltrichlorotriboron nitride. The authors suggest the cyclic structure.

Martius (41) passed hydrogen cyanide gas into cooled liquid boron trichloride and obtained a white solid which changed to a brown liquid at ordinary temperature. He examined it no further but inferred that a molecular compound had been formed. Gustavson (23, 27) found that neither the silver nor the potassium salts of hydrogen cyanide react with boron trichloride.

Substitution of a methyl group for the hydrogen atom in hydrogen cyanide seems to improve the donor properties of the nitrogen atom. A coordination compound between acetonitrile and boron trichloride, $\text{CH}_3\text{CN} \cdot \text{BCl}_3$, has been reported by Nespital and coworkers (46, 61, 62). It is a white crystalline compound melting at 169°C.

Replacement of the hydrogen atom of hydrogen isocyanide with a chlorine atom seems to improve the donor properties of the nitrogen atom. Dry cyanogen

chloride was bubbled through cold boron trichloride and a white crystalline substance resulted which, when analyzed for chlorine, seemed to have the composition $\text{CNCl} \cdot \text{BCl}_3$. Martius listed $\text{CN}_2\text{Cl} \cdot \text{BCl}_3$, but the analysis gave 79.7 and 79.2 per cent chlorine, values which are in good agreement with the formula $\text{CNCl} \cdot \text{BCl}_3$ (41).

Geuther (19, 20) found that nitrogen tetroxide reacted with boron trichloride to produce a solid substance suspended in the liquid phase, while yellow crystals condensed on the walls of the reaction vessel. When the material was distilled after standing for 2 days, volatile yellow crystals condensed in the receiver. Upon exposure to air, the crystals evolved a brownish red vapor. The distillate appeared to contain a solution of the crystals in boron trichloride. The composition of the crystals corresponded to $\text{NOCl} \cdot \text{BCl}_3$. The reaction can be expressed by the following equation:



The crystals were rhombic octahedra or prisms which dissolved in water with production of chlorine, boric acid, and nitric acid. They melted at 23–24°C. forming two liquid layers, the upper of which appeared to be boron trichloride containing a small portion of the molecular compound, while the lower layer consisted of the fused coördination compound and a small quantity of nitrosyl chloride. Upon cooling, the two layers reunited to produce $\text{NOCl} \cdot \text{BCl}_3$. The synthesis of this compound from nitrosyl chloride and boron trichloride has not been reported. The similarity of this compound, $\text{NOCl} \cdot \text{BCl}_3$, to the one previously discussed, $\text{CNCl} \cdot \text{BCl}_3$, is interesting. In both compounds the chlorine atom is attached to the nitrogen atom, the only difference being the oxygen or carbon atoms which are also attached to the nitrogen atom. The carbon, oxygen, or chlorine atom might be the donor atom, but the properties of similar boron trifluoride compounds suggest the nitrogen atom rather than the carbon, chlorine, or doubly bound oxygen. The same property might be expected with boron trichloride.

In view of the numerous compounds containing nitrogen which coördinate with boron trichloride, it appears that the nitrogen atom is able to coördinate regardless of its environment.

B. Phosphorus

Besson (2) observed that phosphine and boron trichloride unite below 20°C. to produce a white solid, $\text{PH}_3 \cdot \text{BCl}_3$. Upon exposure to air, the substance rapidly decomposes, and with water it reacts vigorously producing phosphine, boric acid, and hydrochloric acid. Dissociation of the compound begins at 20°C. under atmospheric pressure. If the products of dissociation are slowly cooled, the compound is obtained in bulky, colorless, highly refractive crystals. Gamble and Gilmont (14) prepared $\text{PH}_3 \cdot \text{BCl}_3$ by the reaction of diborane diphosphine and hydrogen chloride under pressure above 0°C. It is strange that an analogous compound between ammonia and boron trichloride has not been reported.

Tarible (60) reported the formation of a double chloride in the reaction of

chlorine with $P_2I_4 \cdot 2BBr_3$. He gave no formula for the compound, but if one assumes that all of the halogen atoms are displaced, the compound $P_2Cl_4 \cdot 2BCl_3$ results. However, P_2Cl_4 is difficult to prepare and therefore this assumption is questionable.

With phosphorus trichloride, Stieber (56) obtained the coordination compound $PCl_3 \cdot BCl_3$ as a white crystalline solid.

Phosphorus pentachloride-boron trichloride, $PCl_5 \cdot BCl_3$, has been prepared in two ways by Tarible (59). Chlorine displaces the bromine from $PBr_5 \cdot BBr_3$ at ordinary temperature, and the bromine from $PBr_5 \cdot BBr_3$ in the cold. Chlorine is the donor atom in $PCl_5 \cdot BCl_3$, but owing to its relationship to phosphorus trichloride, it is discussed at this point.

Apparently the replacement of the hydrogen atoms by chlorine atoms does not materially affect the donor properties of the central phosphorus atom.

C. Arsenic

Arsine and boron trichloride produce no compound at room temperature or at $0^\circ C$. However, Stieber (56) obtained a white crystalline compound, $AsH_3 \cdot BCl_3$, at $-80^\circ C$. A melting point is unobtainable since the compound dissociates at $-40^\circ C$. with the evolution of arsine.

IV. GROUP VI

Oxygen and sulfur in their compounds are reported as donor atoms to the boron atom of boron trichloride. With such organic compounds as alcohols, ketones, acids, acid anhydrides, and esters, boron trichloride readily splits out hydrogen chloride. Boron trifluoride, in contrast, readily coordinates with such compounds (3). However, coordination compounds with boron trichloride are reported with acid chlorides, phenols, ethers, and dioxane.

A. Oxygen—inorganic

Gaseous and liquid boron trichloride react with water, forming hydrochloric acid and boric acid (10, 11, 38, 72). This reaction is quite different from those observed for the hydrides of the elements of Group V. Dumas (10) observed that a solid hydrate is formed if the quantity of water used is very small. When the hydrate was heated in a stream of hydrogen, hydrochloric and boric acids were obtained. The work of Dumas has never been confirmed (45). On the other hand, boron trifluoride is decomposed by water, producing hydrofluoric and boric acids, but with limited quantities of water is reported to form $BF_3 \cdot H_2O$, $BF_3 \cdot 2H_2O$, and $BF_3 \cdot 3H_2O$ (3).

The existence of the oxychloride of boron, $BOCl$, is very questionable. The percentage composition of this compound is the same as that of a mixture of boric anhydride and boron trichloride, $B_2O_3 \cdot BCl_3$. In the research cited below, no molecular weights were determined, and there is no evidence that either $BOCl$ or $B_2O_3 \cdot BCl_3$ exists, although the authors postulate the synthesis of the oxychloride of boron.

Gustavson (26, 27) stated that equivalent quantities of boron trichloride and

boric anhydride combine, when heated in a sealed tube at 150°C., to form boron oxychloride. He assigned no formula to this compound. The same product is obtained by heating boric anhydride and phosphorus pentachloride at 140–150°C. for 3 to 4 days in a sealed tube. Lorenz (39) tried to confirm this reaction and reported the product to be a mixture consisting of $7\text{B}_2\text{O}_3 + 2\text{BCl}_3$.

Coulner (8, 9) claimed that BOCl_2 is obtained as a by-product in the preparation of boron trichloride. He described it as a yellowish green liquid which decomposes to boron trichloride, chlorine, and boric anhydride upon heating. With water, it hydrolyzes to give chlorine, boric acid, and hydrochloric acid. Lorenz (39) tried to prepare this compound from chlorine, boric anhydride, and charcoal, but obtained, instead, a liquid mixture of boron trichloride and benzene hexachloride. After the liquid mixture was distilled, the oxychloride remained as a white fibrous mass.



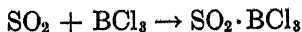
Upon heating this compound, the above reaction is reversed (39). Wöhler and Deville (72) reported that a little boron oxychloride always forms in the reaction vessel in which chlorine reacts with boron to produce boron trichloride. A trace of water in the chlorine or a little boric anhydride in the boron was thought to be responsible for its formation.

More recently, Wiberg and coworkers (51, 67) have proposed that boron oxychloride, BOCl , is one of the products obtained when $\text{BCl}_2\text{OC}_2\text{H}_5$ is heated to 50°C. They did not isolate this compound nor has its existence been proved.

Gustavson (24, 28) discovered that phosphorus pentoxide and boron trichloride produce a crystalline compound when heated to 200°C. in a sealed tube for 2 to 3 days. This molecular compound, phosphoryl chloride-boron trichloride, $\text{POCl}_3 \cdot \text{BCl}_3$, is obtained also from phosphoryl chloride and boron trichloride, and from boric anhydride and phosphoryl chloride when heated in a sealed tube at 150°C. for 8 to 10 hr. It is a rhombic crystalline solid melting at 73°C. Its dissociation pressure and free energy have been determined (6). Upon distillation it decomposes, yielding phosphoryl chloride and boron trichloride. In water or moist air it is converted into phosphoric acid, boric acid, and hydrochloric acid (24, 28). Oddo and Tealdi (48) produced the same compound from boric anhydride and phosphoryl chloride but were unable to confirm its synthesis from phosphoryl chloride and boron trichloride. This latter synthesis should be checked.

Gustavson found that boron trichloride and sulfur trioxide react within 8 hr. at 150°C. in a sealed tube to form sulfuryl chloride and a molecular compound, $\text{B}_2\text{O}_3 \cdot \text{SO}_2$ (25). Recently Luchinskii (40) has found that a white, flaky, gel-like precipitate is obtained when liquid sulfur trioxide is added to cooled boron trichloride. Analysis of the precipitate showed a composition corresponding to $2\text{SO}_2 \cdot \text{BCl}_3$. The compound decomposes at room temperature with the separation of a liquid. Quill (49) and Whittenberg (63) mention the compounds $\text{SO}_2 \cdot \text{BCl}_3$ and $\text{SO}_2 \cdot \text{BCl}_2$. Whittenberg (63) stated that sulfur dioxide and boron trichloride react to form a yellow liquid, which upon exposure to air be-

comes crystalline. The substance was not analyzed, but the suggestion was made that a complex compound forms, according to the following equation:



The compound $\text{SO}_2 \cdot \text{BF}_3$ exists (4). A thermal analysis of the system sulfur dioxide-boron trichloride would be helpful.

B. Oxygen—organic

Whereas a large number of coordination compounds between organic compounds and boron trifluoride have been prepared (3), only a few are known between organic compounds and boron trichloride. Boron trichloride and organic compounds show a strong tendency to lose hydrogen chloride, forming organo-boron chlorides. Possible explanations for such behavior were offered in the introduction of this paper.

(1) *Alcohols*: With alcohols, stepwise substitution of the chlorine atoms of boron trichloride occurs with the formation of boric esters. Berzelius (1) noted that boron trichloride dissolves in alcohol with the evolution of an ether-like odor. Later, Nicklès (47) prepared a "hydrated boron chloride ether" by the reaction of dry hydrogen chloride gas upon an absolute alcohol solution of boric anhydride. Schiff (54) could not confirm this reaction. Instead he obtained a mixture of ethanol, "boric acid ether," ethyl chloride, hydrogen chloride, and water. Cunculer (7, 8, 9) found that allyl alcohol, ethylene glycol, and benzyl alcohol react with boron trichloride with the evolution of hydrogen chloride.

Wiberg and coworkers (65, 66, 67, 69) synthesized the series of compounds ROBCl_2 , $(\text{RO})_2\text{BCl}$, and $(\text{RO})_3\text{B}$ (where R is a methyl or an ethyl group) by the reaction between the alcohol and boron trichloride at $-90^\circ\text{C}.$ to $-60^\circ\text{C}.$ The suggestion is made that the alcohol apparently first forms a coordination compound, $\text{ROH} \cdot \text{BCl}_3$, which later loses hydrogen chloride. These addition compounds have not been isolated (69, 70).

Kinney, Thompson, and Cheney (36) prepared a similar compound, diisooamyloxyboron chloride, $(\text{C}_6\text{H}_{11}\text{O})_2\text{BCl}$, from isoamyl alcohol and boron trichloride.

Fischer and Taurinsch (13) prepared compounds of boron trichloride and benzeneazo-*p*-phenol by carrying out the reaction in carbon disulfide. The compound $2\text{C}_6\text{H}_5\text{N}_2\text{C}_6\text{H}_4\text{OH} \cdot \text{BCl}_3$ decomposes at $166^\circ\text{C}.$, and the compound $3\text{C}_6\text{H}_5\text{N}_2\text{C}_6\text{H}_4\text{OH} \cdot \text{BCl}_3$ decomposes at $158^\circ\text{C}.$ Both compounds precipitated as fine red crystals in the carbon disulfide solution.

(2) *Ketones*: Schumacher (55) reported that boron trichloride reacts with diketone to produce boro-organic salts. Acetylacetone is reported to react with boron trichloride, splitting out hydrogen chloride and forming complex compounds (53). Ketones are reported to coordinate with boron trifluoride (3).

(3) *Acids*: Gustavson (23, 27) treated a mixture of acetic acid and acetic anhydride with boron trichloride and obtained a crystalline compound which hydrolyzed to give boric acid, acetic acid, hydrogen chloride, and acetyl chloride. Meerwein and Maier-Hüser (42) later found that acetic anhydride and boron

trichloride react to give pyroboron acetate $[(CH_3COO)_2B]_2O$ and acetyl chloride. An addition compound is reported by them to be formed when boron trichloride vapor is introduced into cooled acetyl chloride. The compound $CH_3COCl \cdot BCl_3$ separates as long needle-like crystals melting between $-70^\circ C.$ and $-60^\circ C.$ At room temperature the compound completely dissociates, evolving boron trichloride.

In 1928, I. G. Farbenindustrie A.-G. obtained a patent for the use of an organic complex compound, such as formed by acetic acid with boron trifluoride, as a moth-proofing material. The claim that boron trichloride would be useful likewise infers the formation of a complex compound between acetic acid and boron trichloride (32).

(4) *Esters*: An ester, such as ethyl acetoacetate, when treated with boron trichloride evolves hydrogen chloride and forms a complex compound (53). Esters and boron trifluoride readily coördinate (3).

(5) *Ethers*: Ebelmen and Bouquet (12) in 1846 reported a reaction between ethyl ether and boron trichloride in which hydrogen chloride was evolved and a compound "ethyl protoborate" was formed. Similarly, methyl and amyl "protoborates" were formed.

Gattermann (15) first reported the coördination compound between ethyl ether and boron trichloride. He stated that boron trichloride is soluble in ether and forms a crystalline, difficultly fusible, double compound with it. According to Wiberg and coworkers (51, 68, 69, 70) boron trichloride reacts with ethers to form mono- and di-alkyl substitution products. With an excess of methyl ether at $-80^\circ C.$ $(CH_3)_2O \cdot BCl_3$ forms. It melts with decomposition at $76^\circ C.$ to a colorless liquid. Its decomposition yields methyl chloride, BCl_2OCH_3 , and boric anhydride. The ethyl ether compound, $(C_2H_5)_2O \cdot BCl_3$, melts at $56^\circ C.$ to a clear liquid (46).

Comparison of the observed dipole moment of boron trichloride in dioxane with the moment calculated for $C_4H_{10}O_2 \cdot BCl_3$ indicates that coördination of boron trichloride with dioxane is almost complete (37).

C. Sulfur

Ralston and Wilkinson (50) have reported the coördination compound $12H_2S \cdot BCl_3$. The ratio was established by analysis of their product for boron, chlorine, and sulfur. Moissan (44) passed hydrogen chloride over boron trisulfide at $400^\circ C.$ and obtained a boron trichloride and hydrogen sulfide mixture. A study of the system boron trichloride-hydrogen sulfide would determine if a one-to-one coördination compound is formed. The question naturally arises as to whether $H_2S \cdot BCl_3$ exists, since $12H_2S \cdot BCl_3$ and $H_2S \cdot BF_3$ (3) have been reported.

Stock and Blix (57) obtained the compound $B_2S_3 \cdot BCl_3$ as colorless crystals.

V. GROUP VII

Fluorine and chlorine in a few of their compounds are reported as serving as donor atoms to boron trichloride.

A. Fluorine

With triphenylfluoromethane, boron trichloride forms an addition compound of a deep yellow color. It appears to be more stable than the corresponding triphenylchloromethane compound described below. It was not analyzed, so no formula can be assigned to it (64). No other examples were found in which fluorine served as a donor atom. Perhaps this is due to the fact that boron trichloride behaves like a negative element in the formation of coordination compounds, and since fluorine is quite electronegative, coordination is not favored.

B. Chlorine

Chlorine and boron trichloride do not react at room temperature or at -80°C . (58). Graff (21) made a thermal analysis of the system chlorine-boron trichloride. He found no maximum and a eutectic only at 65.5 per cent boron trichloride by weight at -135.4°C . It is evident therefore that elemental chlorine will not donate to the boron atom of boron trichloride.

Graff (22) also studied the system hydrogen chloride-boron trichloride. He found no maximum and a eutectic only at 44 per cent boron trichloride by weight at -134.5°C .

Moissan (44), however, found that chlorine reacts with boron trisulfide to produce sulfur tetrachloride-boron trichloride, $\text{SCl}_4 \cdot \text{BCl}_3$. This compound is a liquid at ordinary temperatures, but melts at -23°C . Moissan mixed one mole of sulfur monochloride and one mole of boron trichloride at -23°C . and obtained no crystals. However, upon introducing chlorine into this mixture abundant crystals formed. Upon raising the temperature to room temperature, the crystals melted to a liquid. The crystals were probably $\text{SCl}_4 \cdot \text{BCl}_3$.

Hoffmann (30, 31) observed that a small quantity of an iridescent green sublimate, the analysis of which corresponds to $3\text{FeCl}_2 \cdot \text{BCl}_3$, was produced when ferroboration was heated in a stream of dry chlorine at a high temperature. It is strange that ferrous chloride should be formed under these conditions. The main product of the reaction was a reddish brown solid which analyzed to be $4\text{FeCl}_3 \cdot \text{BCl}_3$. In a similar manner, manganese boride produced a yellowish-brown product which, when analyzed, appeared to be $2\text{MnCl}_2 \cdot \text{BCl}_3$. In all of these compounds, the chlorine atom is thought to be the donor atom.

Wiberg and Heubaum (64) reported that boron trichloride and triphenylchloromethane react at room temperature and at 0°C . to form a deep yellow addition compound, the analysis of which corresponds to $(\text{C}_6\text{H}_5)_3\text{CCl} \cdot \text{BCl}_3$. When heated to 200°C . it turns a dark brown color but does not melt. When heated to 80°C . under vacuum it distills with decomposition.

Michaelis and Becker (43) found that chlorine decomposes monophenylboron chloride, $\text{C}_6\text{H}_5\text{BCl}_2$, at ordinary temperature to produce a mixture of monochlorobenzene and boron trichloride. At -18°C ., however, monophenylboron chloride absorbs chlorine, producing monophenylboron tetrachloride, $\text{C}_6\text{H}_5\text{BCl}_4$.

This compound is very unstable and when removed from the cooling bath decomposes thus:



On the basis of the first equation, the tetrachloride might be considered as a coördination compound of monochlorobenzene and boron trichloride, $\text{C}_6\text{H}_5\text{Cl} \cdot \text{BCl}_3$; however, $\text{C}_6\text{H}_5\text{BCl}_4$ is favored by the method of synthesis.

VI. SUMMARY

Boron trichloride forms relatively few coördination compounds in comparison with boron trifluoride. No coördination compounds are reported in which the donor atom is a member of Groups 0 to IV in the Periodic Table.

Of the Group V elements, nitrogen, phosphorus, and arsenic in their compounds are reported to be donors to boron trichloride.

In Group VI, oxygen and sulfur in their compounds are reported to form coördination compounds with boron trichloride. Organic oxygen compounds form very few coördination compounds with it. As a rule, the boron trichloride reacts with the organic compound with the evolution of hydrogen chloride and the formation of a substituted boron chloride.

Fluorine and chlorine in their compounds are the only members of Group VII reported to form coördination compounds with boron trichloride.

Only seven elements, nitrogen, phosphorus, arsenic, oxygen, sulfur, fluorine, and chlorine, comprising the upper right-hand corner of the Periodic Table, have been found in their compounds to be donors to the boron atom of boron trichloride, thus forming coördination compounds.

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